ADVANCED GRAPEVINE GENETICS FOR GRAPEVINE IMPROVEMENT



FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

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Table of Contents

Abstract 1

Executive Summary 2

Acknowledgments 4

1 Background 5

1.1 Industry context 6

2 Project Aims 7

3 Methods 7

3.1 Plant Material 7

3.2 DNA profiling 8

3.3 RNA extraction for RT-qPCR 8

3.4 RT-qPCR method and analysis 8

3.5 Phenotyping and evaluation of field grown plants 9

3.6 Micro-scale winemaking 9

3.7 Chemical and sensory analysis of berries and wine 11

3.8 Microvine mutagenesis 11

4 Results & Discussion 12

4.1 Creation of a mutagenized microvine population for forward and reverse genetic studies 12

4.2 Development of SNP markers linked to traits for Marker Assisted Selection (MAS) 14

4.3 Marker assisted selection (MAS) 15

4.4 Evaluation of marker assisted selections in the field 16

4.4.1 Plant vigour 18

4.4.2 Bunch architecture 20

4.4.3 Berry variation 22

4.4.4 Fruitfulness and yield 24

4.4.5 Relationship between vigour and yield 27

4.4.6 Mildew and botrytis resistance in the field 29

4.4.7 Phenology 30

4.5 Grape and wine analysis 36

4.5.1 Micro-scale winemaking 36

4.5.2 Chemical analysis 38

4.5.3 Sensory evaluation 43

4.6 Phenotyping selections in the vineyard and winery 47

5 Outcome & Conclusion 50

6 Recommendations 51

6.1 Future Research Directions 51

6.2 Industry Context and Adoption Scenarios 52

Appendix 1 Communication 54

Appendix 2 Intellectual Property 55
Appendix 3 References 56
Appendix 4 Staff 58
Appendix 5 Other Material and Raw Data 59

Abstract

This project was successful in using advanced genetic approaches for both strategic and applied grapevine research. The methods developed resulted in the creation of a mutagenized grapevine population for gene function studies and the discovery and linking of DNA markers to key agronomic and berry traits. Crucial to the success of the project was the use of microvine material that flowered quickly and had a short generation time. The DNA markers were further developed and used to determine if marker-assisted selection (MAS) was possible for speeding up the process of producing new grapevine varieties. Over 8,000 young grapevine seedlings in a glasshouse were screened with DNA markers for mildew resistance, flower sex and berry colour and over 1,200 that passed the DNA screen were planted in the field for evaluation. The DNA markers accurately predicted the phenotype of the plants in the field. This first generation of powdery and downy mildew resistant selections displayed no evidence of mildew in the field in a no-spray vineyard. To identify elite selections within the 1,200 vines, assessment of agronomic performance and berry and wine attributes was initiated with both white and red selections found to have variation in yield and diverse wine flavours.

Executive Summary

The future competitiveness of the Australian wine industry will depend on reduced input costs, sustainable production methods and a differentiated product that appeals to different markets and changing consumer preferences. Genetic solutions, in the form of new varieties, have been key to overcoming disease problems, increasing yield and producing new products in many crops. Improved grapevine planting material that reduce costs, produce new or improved wine styles and are better adapted to the changing Australian climate will provide industry with new options for maintaining profitability. However the main scientific barriers to producing new winegrape varieties have been the lack of genetic knowledge on what genes control key traits and the long generation time of the grapevine plant making breeding slow and difficult.

The aim of this project was to investigate new genetic approaches for both strategic and applied grapevine research. The objectives of the project were achieved.

One of the strategic objectives was to produce a mutagenized grapevine population. Mutagenized plant populations have proved very useful in other crops for linking genes to traits of economic importance and for producing new varieties. In this project new methods were developed for grapevine mutagenesis using the chemical mutagen EMS and it was confirmed at the DNA level and by changes to the phenotype that plants had been mutated. The development and use of a highly homozygous microvine line contributed to the success of the study as the rapid flowering microvine phenotype allowed two generations of plants to be assessed in a short period of time.

Microvine genotypes were developed in a previous GWRDC project CSP 06/01 and research was continued in this project to develop new microvine germplasm and DNA markers for the genetic dissection of traits. Utilizing different microvine populations and SNP DNA markers the gene change responsible for the fleshless berry locus was identified and a candidate gene controlling flower sex (flower fertility) identified. SNP DNA markers were also successfully linked to traits controlling berry colour and powdery and downy mildew using segregating microvine populations.

The applied objective of the project was to investigate if SNP DNA markers could be used to rapidly create the first generation of new Australian winegrape varieties resistant to downy and powdery mildew suited to Australian conditions. A SNP DNA panel was developed and used for screening seedlings for berry colour, flower sex, mildew resistance and vine stature. This marker-assisted selection (MAS) approach was used to screen over 8,000 young seedlings shortly after germination. Based on the DNA marker analysis over 1,200 were identified as being mildew resistant and having perfect flowers and either white or red berries. The 1,200 selections were planted in a no-spray field in the Barossa Valley for evaluation. When the vines

flowered they were scored for flower sex and berry colour and it was found that the DNA panel accurately predicted the phenotype of the plant for the traits screened. This is a significant advance on current grapevine improvement programs where large numbers of vines are planted in the field and a period of years elapse before flower and berry traits can be scored.

The selections planted as single vines (617 white selections and 627 red selections) in a no-spray vineyard in the Barossa Valley showed no evidence of downy or powdery mildew in the field on leaves or berries during the evaluation period starting in 2010. However the selections were not resistant to botrytis. It was found that there was large variation for vine vigour, yield, fruitfulness (inflorescence number on a cane), bunch architecture, bunch size, berry size and berry maturity (harvest) date. Chemical analysis of berries was carried out on those that produced fruit during the evaluation period and variation was found for °Bx, pH, TA, tartaric acid, malic acid and YAN. The sensory evaluation of micro-scale white wines by commercial winemakers identified a wide range of flavour profiles in the selections with some selections being scored as of high interest by some of the winemakers.

The successful demonstration in this project of the advantages of MAS to greatly improve the efficiency of grapevine breeding indicates that it will become an important long-term tool for future grapevine breeding of both scions and rootstocks. The outcomes of other projects linking genes and DNA markers to traits of economic importance can be incorporated into the existing SNP panel developed in this project to screen for additional traits at the seedling stage.

Initial evaluation of the selections for field performance and berry and wine attributes have been promising with some selections having high yields and some white wines being scored as of high interest by commercial winemakers. Further research is required to characterise in greater detail the wine attributes and agronomic performance of the selections over several seasons especially traits strongly influenced by the environment.

A proposed pathway to adoption strategy for elite selections identified at the Barossa site is to develop collaborative regional trials with industry and researchers. This would allow growers and winemakers to see the performance of new varieties in their own region and make informed decisions based on both vine and wine traits. This strategy would also assist overall evaluation of selections with data for different environments obtained in a single year for the same selection instead of collecting data over a number of years at a single site.

The research results and outcomes described in this project report would not have been possible without the valuable contributions of the people listed in the Acknowledgement section.

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1 Background

International competitiveness of the Australian wine industry will depend on reduced input costs, sustainable production methods and a differentiated product that appeals to different markets and changing consumer preferences. To remain competitive, it is likely that genetic solutions will become increasingly important through the use of improved planting material rather than relying solely on additional management techniques that lead to increased costs. The foundation of genetic improvement is an understanding of what genes control key traits through genomic studies and applying this information to rapidly select improved varieties by advanced genetic methods.

The major problems that have hindered development of improved grapevine material has been the lack of knowledge on what genes control key traits and the biology of the plant itself having a long generation time and large space requirements in the field.

In a previous GWRDC project (CSP 06/01 “Development of a rapid genetic system for linking traits to genes”) new rapid systems for grapevine genetic studies were developed based on a new grapevine type called the microvine (Chaib *et al*., 2010). New microvine lines suitable for rapid evaluation of transgenes targeted to the berry to determine gene function were developed and it was demonstrated that introduced genes expressed in the berry could be evaluated in approximately one year which is 2-5 times quicker than existing grapevine systems. The development of microvine populations to link DNA markers and genes to traits by genetic mapping was demonstrated through the use of rapid breeding methods with microvine populations flowering and producing fruit in less than one year for berry phenotyping and genetic mapping. This was a significant advance over existing systems that require 3-5 years to produce progeny populations suitable for genetic mapping of berry traits. Inbred microvine lines were also produced that would have taken 20-25 years using traditional varieties and breeding methods were also produced.

The use of the microvine system and linking DNA markers and genes to traits has the potential to rapidly increase our knowledge of gene function for grapevine improvement. The development of these fast grapevine genetic systems has also provided the foundation to develop fast breeding methods and to begin to apply advanced genetic approaches to winegrape improvement. Successful development would see a reduction from the traditional 25-30 year period required for breeding, evaluation and release to industry of a new variety to 10 years or less.

To maintain Australia’s competitive advantage in strategic and applied grapevine genomic research this project investigated the further advancement of rapid microvine based systems by development of a mutant microvine population for gene

function studies. The project also aimed to improve the future competitiveness of the Australian wine industry by applying knowledge from genomic studies to an advanced genetic breeding technique known as marker-assisted selection (MAS) to rapidly create the first generation of Australian wine grape varieties resistant to downy and powdery mildew for evaluation under Australian conditions.

1.1 Industry context

The research was closely aligned with a number of GWRDC program areas; grapes for purpose, sustainability of the industry, environment & communities and smart science, practical solutions. The wine industry is facing a number of difficult challenges world-wide including the very high usage of fungicides compared to other crops to control powdery and downy mildew (Eurostat, 2007). Potential economic benefits of this research project will be reduced production costs with mildew resistant varieties requiring fewer inputs (e.g. chemicals, fuel) due to fewer spray applications. Unlike traditional cultivars such as Chardonnay or Cabernet Sauvignon these new wine varieties will have the additional advantage in that they can be protected by intellectual property strategies to restrict their availability to a specific country like Australia or specific wine companies (Thomas *et al*., 2007). Regional communities benefit from economically sustainable industries and new wine grape varieties that have lower production costs and produce a desirable marketable product may assist the Australian wine industry maintain a competitive advantage in the global market. Potential environmental benefits of new disease resistant varieties include a reduction in the need for chemical sprays to control mildew, reduced fuel and energy consumption and reduced soil compaction due to less tractor activity in vineyards.

2 Project Aims

Functional genomics involves the development and use of methods to determine the function of genes and the linking of genes to key traits. Knowledge of which genes control key traits is currently very poor for grapevine. Exploitation of grape genomic information by Australian grapevine researchers and industry will depend on the ability to quickly identify and confirm the function of genes that are involved in key traits. An understanding of gene function is an important first step in applying this information for the benefit of the industry. Research progress in project CSP06/01 has solved 2 of the 3 major impediments for rapid grapevine gene function studies. Transgenes can be rapidly tested and microvine populations rapidly produced for genetic mapping. The remaining essential genetic "tool" for gene function studies is the creation of mutants by mutagenesis of near homozygous inbred lines to link a trait to a mutated gene.

The project objectives were:

1. Complete existing CSP 06/01 objectives in 2009/10 to finalise development of genetic material, germplasm and DNA markers suitable for rapid genetic analysis of traits

2. Develop a mutagenised microvine population from a selected homozygous inbred microvine line to link traits to genes

3. Apply advanced genetic techniques and knowledge obtained from functional genomic studies to rapidly create the first generation of new Australian winegrape varieties resistant to downy and powdery mildew suited to Australian conditions.

3 Methods

3.1 Plant Material

Microvine and wild-type genotypes were selectively crossed to produce progeny populations for DNA marker evaluation using SNP (Single Nucleotide Polymorphism) markers. Seed germination was essentially as described previously (Chaib *et al*., 2010). Microvine progeny populations were maintained in the glasshouse or a growth room for collecting phenotype data.

Wild-type progeny plants were maintained in a field at Nuriootpa, South Australia for the collection of phenotype data, and performance evaluation. The vines were grown in no-spray blocks that received no sprays for downy mildew, powdery mildew or botrytis. Prior to field planting the seedlings were grown to wire height in a glasshouse over a period of 4 months and planted in Spring.

3.2 DNA profiling

Small samples (approx. 0.1 g) of young leaf were collected from plants. DNA was extracted using standard procedures with commercial kits or by an automated DNA extraction platform at the AGRF.

For SNP DNA profiling the MassARRAY iPLEX assay with matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) detection (Sequenom, http://www.sequenom.com/) was used essentially as previously described (Chaib *et al*., 2010) . The SNP genotyping was performed by AGRF.

For DNA marker design and testing, DNA sequencing was performed using standard procedures and the sequence information analysed using the software CLC Genomics Workbench. Selected DNA sequences targeting specific SNPs were submitted to AGRF for primer design and genotyping. The SNP genotyping results were analysed for Mendelian inheritance and to identify SNPs that were linked to specific traits.

3.3 RNA extraction for RT-qPCR

RNA was extracted using a Spectrum plant total RNA kit (Sigma, www.sigmaaldrich.com) as recommended by manufacturer, including DNase treatment (Qiagen, www.qiagen.com).

3.4 RT-qPCR method and analysis

For real-time quantitative PCR (RT-qPCR) first strand cDNA was synthesized from 1 µg of total RNA using 200U of Superscript III cat #18080-085 (Invitrogen), 2.5 µM Oligo (dT)20 primer and 500 µM dNTPs and incubated at 50 °C for 1 hour in a total of 20 µl. The resulting first strand cDNA was diluted 1/20 with water for RT-qPCR. LightCycler 480 SYBR Green 1 Master 2x concentration cat # 04 887 352 001 (Roche) was used in the RT-qPCR assay at a 1x concentration along with 0.5 µM gene specific primers and 2 µl of first strand cDNA in a final reaction volume of 15 µl. Water and no reverse transcription negative controls were included in the RT-qPCR reactions. PCR amplification was done in a Rotor-Gene RG-3000 (Corbett Research) or LightCycler 480 II (Roche), 95 °C for 10 minutes then 50 cycles of 95 °C 20 seconds, 58 °C 20 seconds, 72 °C 20 seconds and then a final step of 72 °C for 5 minutes. All primer pairs were examined for the amplification of a single product of the expected size and sequence. The presence of a single product was confirmed by melt-curve analysis using the same software and also by gel electrophoresis. The genes were also confirmed by sequencing. Target genes and reference genes expression levels were obtained from the Ct values generated by the software.

Relative gene expression levels between different tissue samples for each gene were determined using Biogazelle qbasePLUS2 software (Integrated Sciences).

Data was normalised using three reference genes, VvUbiquitin (BN000705), VvEIF (XM_002278083) and Actin1 (XM_002282480) genes.

3.5 Phenotyping and evaluation of field grown plants

Single vines were evaluated each season, over a minimum of 2 seasons for a number of important agronomic traits; flower sex, inflorescence number per cane, fruitfulness of spur pruned canes, bunch number, berry colour, date of berry maturity (22 °Bx (degrees Brix) for whites and 23 °Bx for reds), bunch type, bunch weight and total yield. Vine vigour was measured using trunk girth at the irrigation line two years after planting.

A powdery mildew detached leaf assay similar to that previously described (Donald *et al*., 2002) was used to determine resistance to powdery mildew.

3.6 Micro-scale winemaking

Micro-scale winemaking was undertaken using volumes from 160 ml to 500 ml for white berries and 1300 g ferments were done for red berries.

White wine was made for the 2011 and 2012 vintage as follows. Frozen grape bunches were de-stemmed into a sealable plastic bag. Sulphur dioxide (SO_2) at 50 ppm was added as a Potassium Metabisulfite (PMS) solution 100 mg/ml with addition of 285 μl to 285 g of berries. Oxygen was removed from the bag and berries were allowed to thaw before they were crushed by hand within the plastic bag. The must was transferred into 250 ml centrifuge bottles and centrifuged at 15,316 g for 5 minutes at 4 ^{o}C in a Beckman JA14 rotor. The resulting supernatant (160 ml of clear juice) was transferred to a fermentation flask. Oxygen was removed from the flask by passing Argon gas through the airlock. The fermentation vessel was transferred to 18 ^{o}C and left for 2 hours for the temperature to equilibrate. Yeast Assimilable Nitrogen (YAN) was added at 200 ppm by adding 0.942 g of Diammonium Phosphate (DAP=21.2% YAN) per litre of juice. The pH of the juice was measured from a 1 ml sub sample (prior to DAP addition) using the Oenofoss^{TM} (FOSS, Denmark) using the must parameter. Yeast (PDM/EC1118 prepared using the manufacturer's instructions - MAURIVIN) was added at 1% juice content (1.6 ml of Yeast culture). The ferment was then incubated with constant stirring 18 ^{o}C for 48 hours and then 15 ^{o}C until the end of ferment. Sugar levels were measured every 48 hours to keep track of fermentation until day 5 after which it was then measured daily. When sugar levels were <2.5 g/L, additions of PMS (120 mg/L), Ascorbic Acid (50 mg/L) and Copper Sulphate (1 mg/L) were added to the wine. The wine was then centrifuged at 4,500 g in an A-4-81 rotor. The resulting supernatant (wine) was transferred to 6 x 20 ml vials. These vials were filled to the brim to prevent air spoilage and stored at 0 – 4 ^{o}C.

White wine was made for the 2013 vintage as follows. Frozen grape bunches were de-stemmed into a sealable plastic bag. Sulphur dioxide (SO_2) at 50 ppm was added as a Potassium Metabisulfite (PMS) 100 mg/ml solution (585 μl for 585 ml of pre-calculated juice) to 900 g of berries. Argon gas was added to the plastic bag of de-stemmed berries to remove oxygen and allowed to lay flat for 90 minutes in order for the berries to thaw out. Once thawed the berries were crushed by hand within the plastic bag. The must was transferred to three 250 ml centrifuge bottles and centrifuged 15,316 g for 5 minutes at 4 ^{o}C in a Beckman JA14 rotor. The resulting supernatant (500ml of clear juice) was transferred to a 500 ml Schott bottle. The same sub sample of juice was used for sugar determination as described in (Boehringer-Mannheim, 1989). The 500 ml Schott bottle was transferred to 18 ^{o}C and left for 2 hours for the temperature to equilibrate. Yeast Assimilable Nitrogen (YAN) was added at 200 ppm by adding 0.942 g of Diammonium Phosphate (DAP = 21.2% YAN) per litre of juice. Polyvinylpolypyrrolidone (PVPP) was added at 260 ppm by adding 260 mg PVPP per litre of juice. The pH of the juice was measured from a 1 ml sub sample (prior to DAP addition) with an Oenofoss^{TM} (FOSS, Denmark) using the must parameter. The pH was adjusted to 3.4 using Tartaric acid (1 g of Tartaric acid per 1 litre of juice for a 0.1 pH value drop). Yeast (PDM/EC1118 prepared using the manufacturer's instructions - MAURIVIN) was added at 1% juice content (5 ml of Yeast culture). The ferment was then placed onto a shaking platform for shaking at 100 rpm for 2 minutes twice a day at 18 ^{o}C for 48 hours and then 15 ^{o}C until the end of ferment. Sugar levels were measured every 48 hours to keep track of fermentation until day 5 from which it was measured daily. When sugar levels were <2.5 g/L the wine was racked using Argon gas to push the wine out into a new clean 750 ml bottle through a long stainless steel pipe that reached the bottom of the bottle containing the wine. The pipe came through the bottle opening through an airtight bung and was attached to the Argon cylinder by a plastic tube. The wine was pushed through gas tubing that also entered the bottle through the same bung and into the new bottle. Care was taken to avoid transferring any sediment to the new bottle. The pH was checked and re-adjusted to 3.4 if needed and SO_2 was added at 50 ppm and Copper sulphate at 1 mg/L. The wine was gently mixed, filled with sterile marbles to reduce headspace and filled with Argon gas to remove oxygen. The wine was left at 4 ^{o}C for 1 week. The wine was then filtered through a sterilized Waterra FHT-45 ground water 0.45 micron filter (Air-Met Scientific) into 100 ml and 50 ml amber glass bottles and sealed with Celloseal (PS22 pressure sensitive styrene foam) wadded tamper proof lids (Cospak). The bottle caps were sealed with wax to avoid air spoilage and the final wines were stored at 16 ^{o}C.

Red wine was made for the 2013 vintage as follows. Grape bunches were de-stemmed into a sealable plastic bag. Sulphur dioxide (SO_2) at 50 ppm was added as a PMS 100 mg/ml solution (850 μl for 850 ml of pre-calculated juice) to 1.3 kg of berries. The de-stemmed grapes were laid flat and gently crushed by hand within the plastic bag. The resulting must was poured into the fermentation vessel (modified

Bodum Chambord Coffee Press 1932-16) and gently plunged to allow some juice to cover the crushed berries. The pH of the juice was measured from a 1 ml sub-sample with an Oenofoss™ (FOSS, Denmark) using the must parameter. The pH was adjusted to 3.4 using Tartaric acid (1 g of Tartaric acid per 1 litre of juice for a 0.1 pH value drop). The same sub-sample of juice was used for sugar determination as described in (Boehringer-Mannheim, 1989). The fermentation vessel was transferred to 22°C and left for 2 hours for the temperature to equilibrate. Yeast (PDM/EC1118 prepared using the manufacturer's instructions - MAURIVIN) was added at 2% juice content (17 ml of Yeast culture). Yeast Assimilable Nitrogen (YAN) was added at 200 ppm by adding 0.942 g of Diammonium Phosphate (DAP=21.2% YAN) per litre of juice. Sugar levels were measured daily to keep track of fermentation. When sugar levels were <2.5 g/L the ferment was pressed by applying a 4 kg weight on top of the plunger for 5 minutes. The fermented liquid was poured into a clean clear 750 ml glass wine bottle. The headspace was filled with Argon gas to remove oxygen. The wine was left at 4 °C for 24 hours. The wine was racked using Argon to push the wine out into a new clear 750 ml wine bottle through a long stainless steel pipe that reached the bottom of the bottle containing wine. The pipe came through the bottle opening through an airtight bung and was attached to the Argon gas cylinder by a plastic tube. The wine was pushed through gas tubing that also entered the bottle through the same bung and into the new bottle. Care was taken to avoid transferring heavy sediments to the new bottle. SO_2 was added at 50 ppm and the pH was checked and re-adjusted to 3.4 if needed. The wine was gently mixed, filled with sterile marbles to reduce headspace and filled with Argon gas to remove oxygen. The wine was left at 4 °C for 2 weeks. The wine was then filtered through a sterilized Waterra FHT-45 ground water 0.45 micron filter (Air-Met Scientific) into 200 ml and 100 ml amber glass bottles and sealed with Celloseal (PS22 pressure sensitive styrene foam) wadded tamper proof lids (Cospak). The bottle caps were sealed with wax to avoid air spoilage and the final wines were stored at 16 °C.

3.7 Chemical and sensory analysis of berries and wine

For berries at harvest an OenoFoss™ machine (FOSS, Denmark) was used to measure degrees Brix, pH, Total Acidity (TA) to pH 7.0, Malic acid, Tartaric acid, density, Yeast Assimilable Nitrogen (YAN), Alpha amino acids, Ammonia, Gluconic acid and Volatile acidity (VA) of the juice. At the end of fermentation the OenoFoss was used to collect information on ethanol concentration, pH, VA, TA, Malic acid, density and glucose and fructose concentrations.

Sensory assessment of the wines was done by experienced winemakers and scored for a number of attributes including aroma intensity, acidity, flavour, potential use and interest level.

3.8 Microvine mutagenesis

This section of the final report is commercial in confidence and will not be available until after June 2015. If you have any questions, please contact the GWRDC.

4 Results & Discussion

There are two major approaches to link DNA markers and genes to traits: either a forward or reverse genetic approach. In a forward genetic approach a unique phenotype is identified and then characterised to identify the genetic change that is responsible for that phenotype. The reverse approach involves starting with a known gene and then studying the phenotype of the gene. A common forward genetic approach is to use genetic mapping to identify the gene responsible for a phenotype while the creation of a mutagenized population is often used to produce mutants and then either use a forward genetics approach to pick a mutant phenotype and identify and characterise the responsible genetic change or screen the mutant population in a reverse genetic approach to look for mutations in a specific gene and then study the phenotype to determine the function of the gene. The other common reverse genetic approach in plants is to study the function of a known gene by creating transgenic plants that either over-express or have reduced expression of the gene.

Once a gene is linked to a trait by either forward of reverse genetic approaches DNA markers can then be developed for marker-assisted selection (MAS) to breed new grapevine varieties with the desired traits in a more efficient manner than traditional breeding approaches.

4.1 Creation of a mutagenized microvine population for forward and reverse genetic studies

The highly homozygous grapevine line 06C008V0010 was used for mutagenesis experiments as this genotype would permit mutations in the DNA to be more easily identified compared to the use of a heterozygous line.

Preliminary experiments were carried out with EMS treatment of seeds. EMS treatment of seed is a standard mutagen treatment in other plant species (Myles *et al*., 2010). To maximise the number of mutations in a genome it is desirable to treat tissue with a high level of mutagen. The high mutagen level results in a high death rate requiring the treatment of thousands of seeds (Bacilieri *et al*., 2013; McMichael *et al*., 2013). It was found that EMS treatment of grapevine seed resulted in poor germination rates and seedling survival (Table 1). This was compounded by the typical low germination rate of untreated seed.

Table 1. EMS treatment of grapevine seed

EMS concentration	Seed treated	Germinated Seed	Surviving seedlings
0%	338	136	95
0.1%	230	75	14
1.0%	230	14	0
3.0%	337	0	0

The disappointing outcome of EMS seed treatment and the requirement for many thousands of seed for treatment necessitated the development of a new method for grapevine mutagenesis.

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4.2 Development of SNP markers linked to traits for Marker Assisted Selection (MAS)

Development of DNA markers linked to a trait requires germplasm containing the trait of interest, genetic material segregating for the trait of interest (usually populations derived from a controlled cross) and a DNA marker type like Single Nucleotide Polymorphisms (SNPs) that can be used in a high throughput assay. To complete the project objective continuing on from CSP 06/01, microvine populations from controlled crosses were produced and SNP DNA markers developed for screening the grapevine genome and identifying SNP markers linked to traits of interest.

The SNP marker assay chosen was the Sequenom MassARRAY iPLEX assay as it was suitable for both genome-wide screening of hundreds of markers and for producing small DNA marker panels composed of specific markers for MAS.

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4.3 Marker assisted selection (MAS)

Based on the results reported in section 4.2, a SNP marker panel was developed and tested to screen 4 traits (flower sex, berry colour, mildew resistance and plant stature) in one Sequenom MassARRAY iPLEX assay containing 8 DNA markers (2 markers per trait). Primer design was optimised for the SNP panel to multiplex all 8 markers in one PCR reaction. The final SNP panel designated GrapeSet1.1 was; flower sex (markers VVMT_66.2 and VMT_137.1), berry colour (markers VVMT2.2 and VVMT4.2), mildew resistance (markers VVMT14.1 and VVMT16.2) and vine stature (markers Vvgai_05.1 and VvGAI.1).

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4.4 Evaluation of marker assisted selections in the field

The selections were planted in the Barossa Valley, South Australia. Two plantings were undertaken, 332 white selections were planted in block 39 in Spring 2009 and in the 2010 Spring 627 red selections and the remaining 285 white selections were planted in block 37. The blocks were adjacent to each other and the vines were irrigated and fertilised to allow the vines to exhibit their full potential under local conditions. No sprays were applied for mildews during the term of the evaluation. The selections were planted and evaluated as single vines on own roots and trained to four cordons.

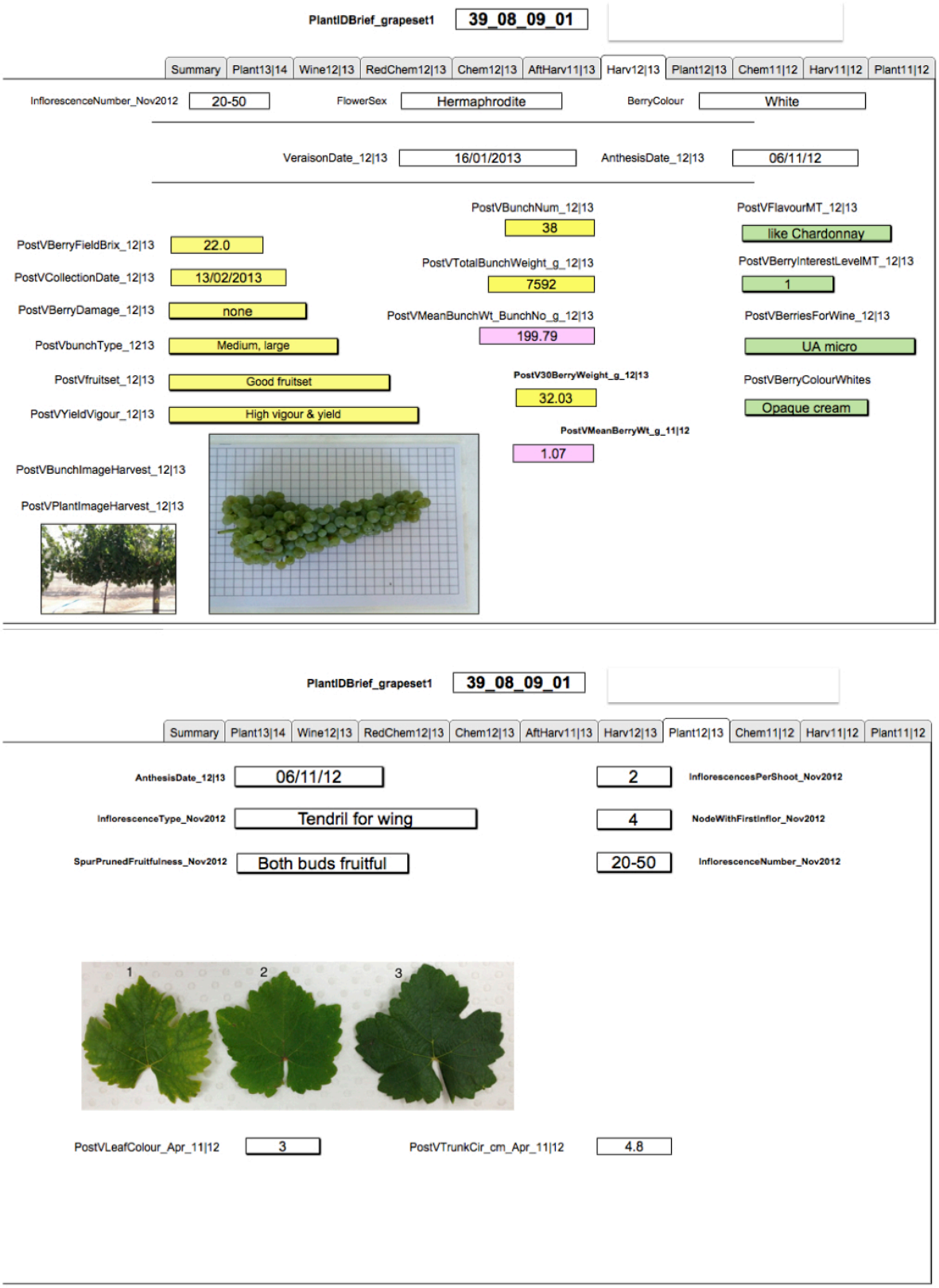
Many selections flowered and produced fruit one year after planting. In block 39, 195 (59%) flowered in Spring 2010 from which fruit was collected in 2011. In block 37, 72% selections flowered and produced fruit in Spring 2011. By the second year after planting the selections in both blocks were producing flowers and fruit from 99% of the selections.

A database was developed to collected data in the field using ipads (Fig. 11). The same database was extended for the collection of chemical data of berries and wine and also for wine sensory analysis. The traits scored in the field are listed in Table 9. For most traits, data were collected for all plants from their first flowering season but due to seasonal differences and different rates of individual plant growth the data presented in the following sections are from season 2012-13 unless otherwise indicated.

Table 9. Data collected in the field for the selections

Plant Growth & vigour	Fruitfulness & yield	Bunch architecture	Berry	Phenology
Trunk circumference	Inflorescences per shoot	Inflorescence type	Berry damage (shrivel, botrytis)	Anthesis date
Leaf colour after harvest	Node with first inflorescence	Bunch type	Average berry weight	Veraison date
Visual assessment of yield and vigour	Inflorescence number	Bunch image	Berry flavour	Harvest date (22 °Bx for whites, 23 °Bx for reds)
Plant image	Inflorescences on spur pruned canes			
	fruitset			
	Bunch number			
	Average bunch weight			
	Total yield			

Figure 11. Traits scored in the field. Screen capture of data entered into database using an ipad.

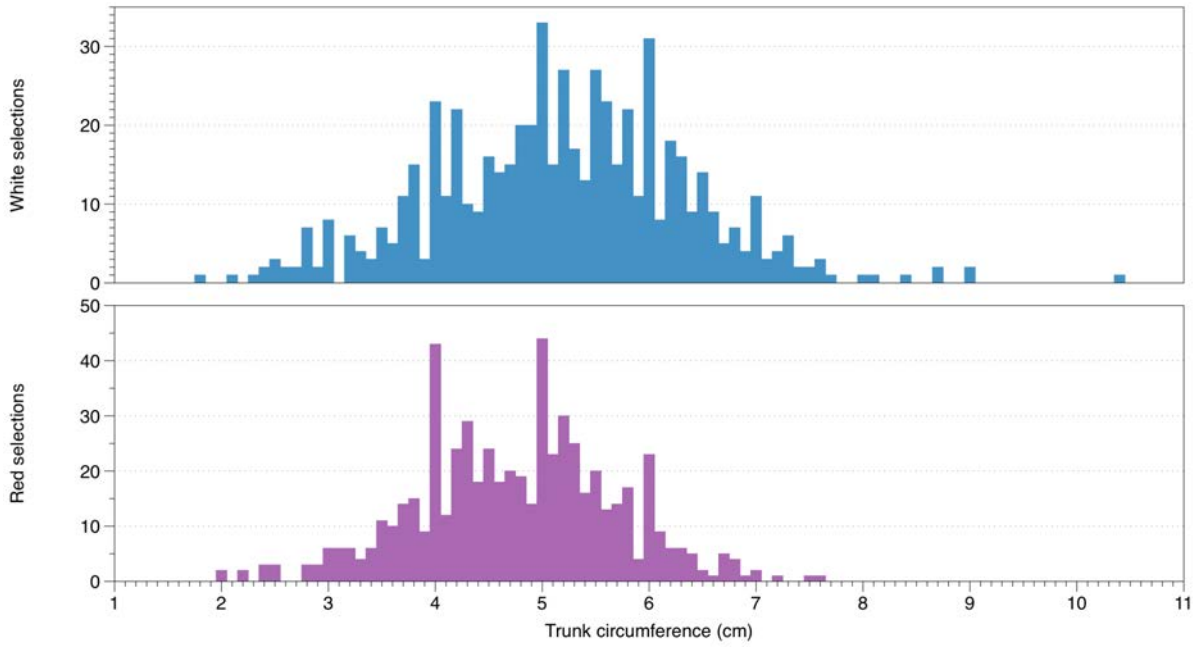


4.4.1 Plant vigour

The majority of the selections produced four cordons and fruited, however 1.5% showed weak growth and either died or produced no fruit. To measure plant vigour two traits were looked at: the circumference of the trunk at the level of the irrigation line after two years of growth and a visual evaluation of leaf greenness at the end of the season after harvest. Pruning weights of canes were not used due to the large number of selections and the young age of the selections.

Trunk circumference ranged from 1.8 - 10.4 cm (Fig. 12). The white selections had the largest trunk circumference compared to the red selections (Fig. 12). Trunk circumference is an indicator of vegetative plant vigour and the ability of the plant to send sugar from the leaves to vegetative organs for storage and growth.

Figure 12. Trunk circumference of white and red selections.



Chlorophyll content of leaves is often an indicator of the health of a plant and the ability to cope with stress (water). At the end of the harvest the leaves of the selections were visually assessed for chlorophyll content on a scale from 1-3 (Fig. 11). In an initial experiment using selections in block 39 in 2012 a relationship was found between leaf colour and trunk circumference (Fig. 13). Selections with dark green leaves (3) had a medium trunk circumference of 5.95 cm while selections with the least green leaves (1) had a medium trunk circumference of 3.3 cm (Fig. 13). The data suggests that there may be a relationship between the ability of a grapevine plant to maintain healthy leaves and high chlorophyll content throughout the growing season and the resulting vigour and growth of the plant as measured by trunk circumference (Fig. 13, 14). Further investigation of this relationship will be necessary using a quantitative measure of leaf chlorophyll content.

Figure 13. Whisker box plot of 329 white selections grouped into leaf colour after harvest and the trunk circumference in each class.

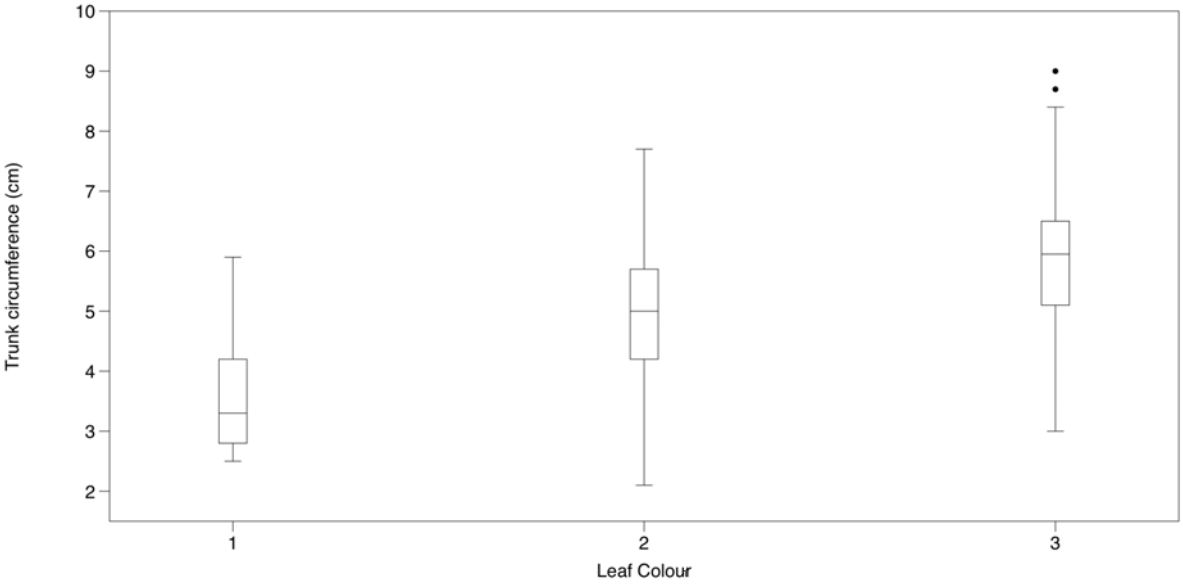


Figure 14. Vigour of selections. Examples of plants grouped into leaf colour 1 (top) and leaf colour 3 (bottom).



4.4.2 Bunch architecture

Bunch architecture is determined largely by inflorescence development prior to anthesis (Shavrukov *et al*., 2004). A more open bunch will reduce contact between berries and allow greater airflow around the berries reducing the humidity after a rain event. These attributes assist in the reduced incidence of botrytis infection and berry damage (Shavrukov *et al*., 2004). Data were collected to determine the variation with the selections.

The variation observed in bunch openness (tight, medium, open) and bunch size (small, average, large) was similar between the white and red selections (Fig. 15). Tight bunches, where the berries are pressed against each other, would be expected to produce an environment more favourable to botrytis infection and growth compared to bunches that were classified as medium in openness or those that were open (Fig. 16).

Figure 15. Bunch type variation for red and white selections. White (588) and red (583) selections scored in 2013.

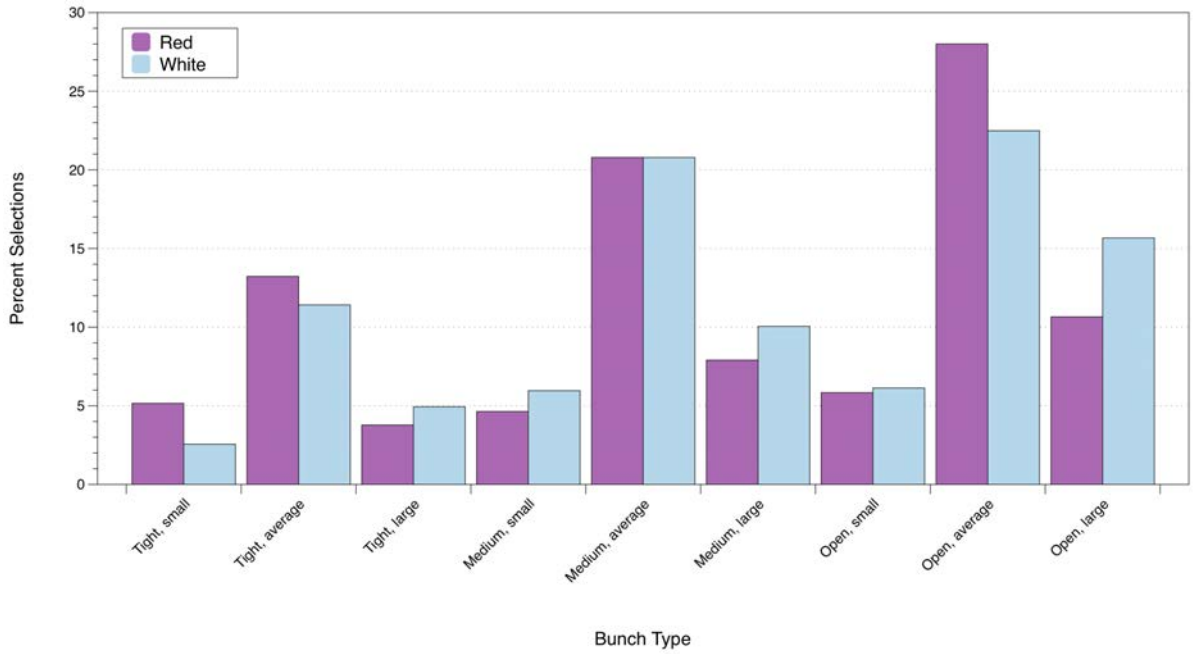
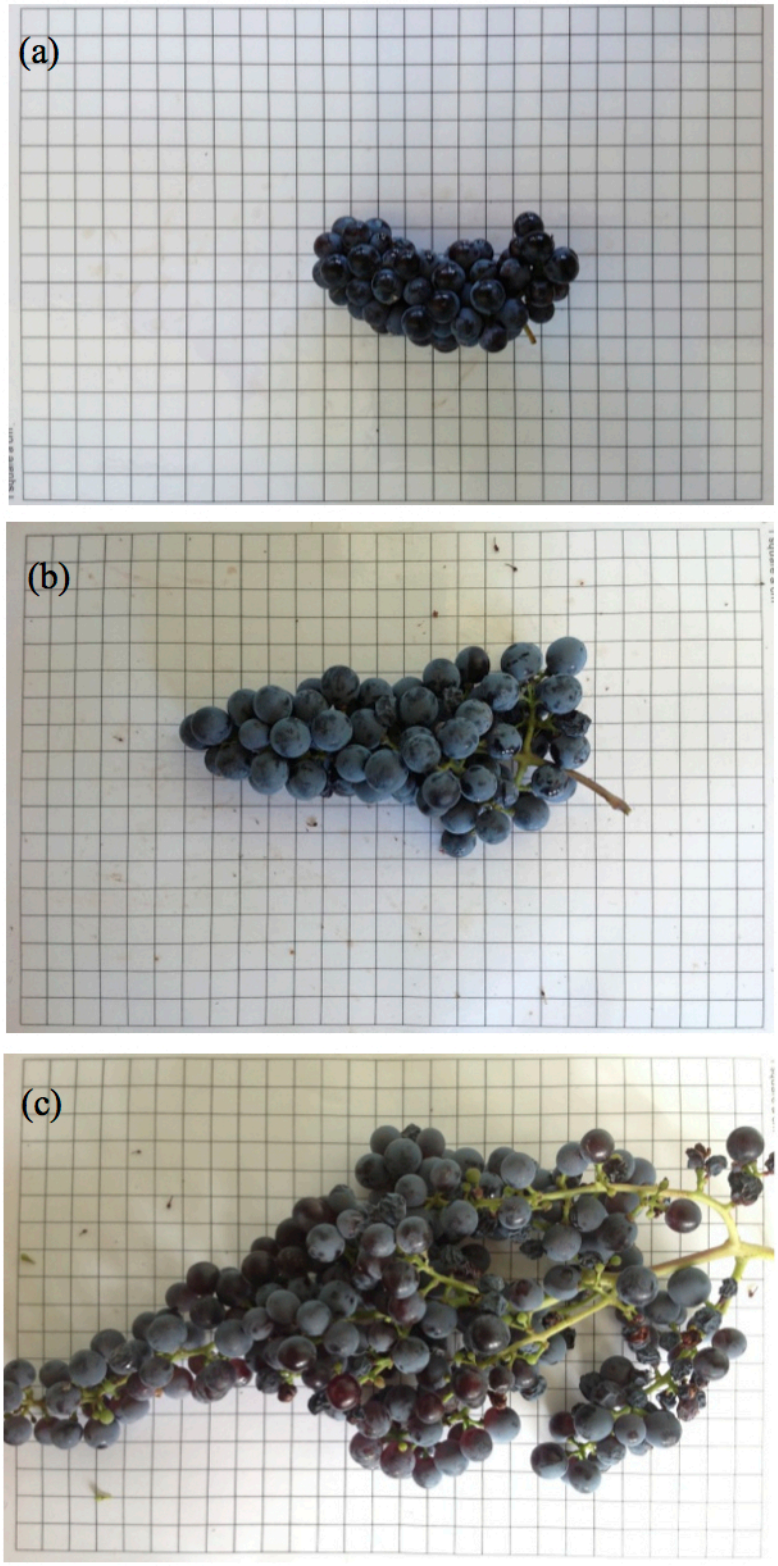


Figure 16. Examples of different bunch architecture and size within the selections. (a) Tight, small bunch, (b) Medium openness and average size bunch, (c) Open, large bunch. Selections photographed in 2013. Grid is 1 cm^2.



The study of fruitful inflorescence wings found that 389 white and 329 red selections developed inflorescences that had a wing with flowers. The presence of a fruitful wing may contribute to increased bunch weight and final yield although bunches per vine and bunches per cane is the main driver of yield per plant.

The large variation in bunch type observed for both white and red selections suggests that a future detailed quantitative genetic study investigating bunch architecture within these populations may assist in a better understanding of the genetic control of bunch architecture and eventually lead to the identification of loci and genes controlling the openness of bunches. To assist in the selection of elite individuals, those selections with tight bunches could be culled (discarded) as they would be more susceptible to damage by botrytis and other types of bunch rot that thrive in a high humidity bunch microclimate.

4.4.3 Berry variation

Berry size is a minor component of fruit yield and may also contribute to juice yield after crushing. Berry size is under genetic control but influenced by the environment and water application during berry development (management) and seed number (Carmona *et al*., 2008). To investigate if there was variation in berry size and extractability of juice from berries data was collected for these traits.

There was variation in berry weight (size) across both white and red selections (Fig. 17). The median berry weight was 0.877 g and 0.817 g for white and red selections respectively. Juice extractability was compared to berry size and a weak non-significant correlation (R^2 = 0.2663) was found (Fig. 18).

There was poor correlation (R^2 = 0.1384) between two years for juice extractability (Fig. 19). This could be due to seasonal variation or because different scale micro-scale ferments were used for 2012 (185 g of berries) and 2013 (900 g of berries).

The selections were subjectively scored at harvest in the field for berry damage. No damage was observed for 67% of the white selections and 73% of the red selections at the 2013 harvest. High levels of shrivel was observed for 4% and 4.5% of white and red selections respectively. Berry shrivel can be due to environmental effects (e.g. high sun exposure), disease (botrytis) or may be under genetic control. It has been found that the variety Shiraz exhibits high levels of cell death in the berry contributing to berry shrivel (Fuentes *et al*., 2010). Sultana and other varieties are well known in industry for susceptibility to rain induced berry splitting followed by shrivel as berry sugar levels increase (Considine and Kriedeman, 1972).

Figure 17. Berry weight variation for white and red selections. 30 berries were sampled from each selection at harvest, weighed and the mean berry weight calculated. 2013 season data.

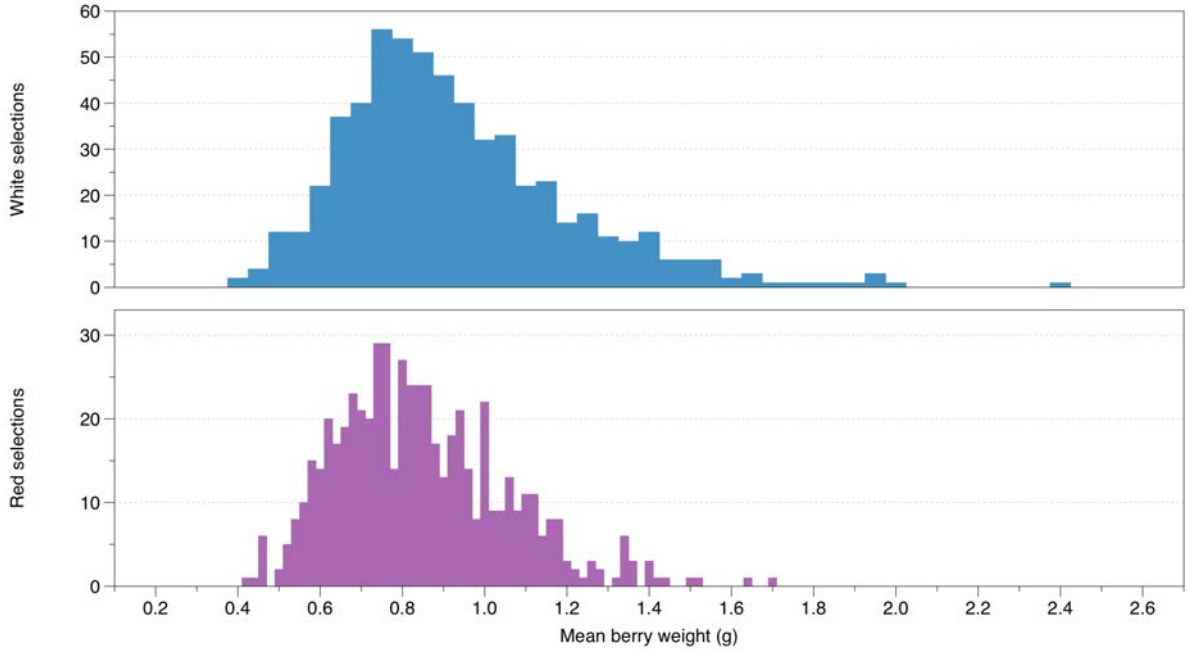


Figure 18. Correlation of juice extractability and berry weight for 90 white selections. 2013 data.

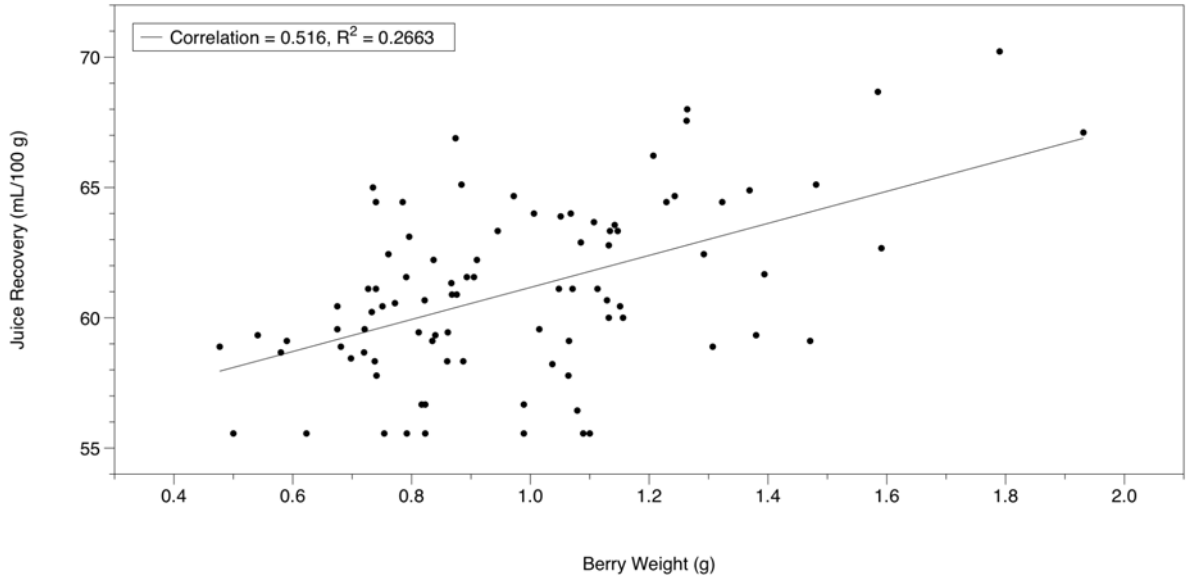
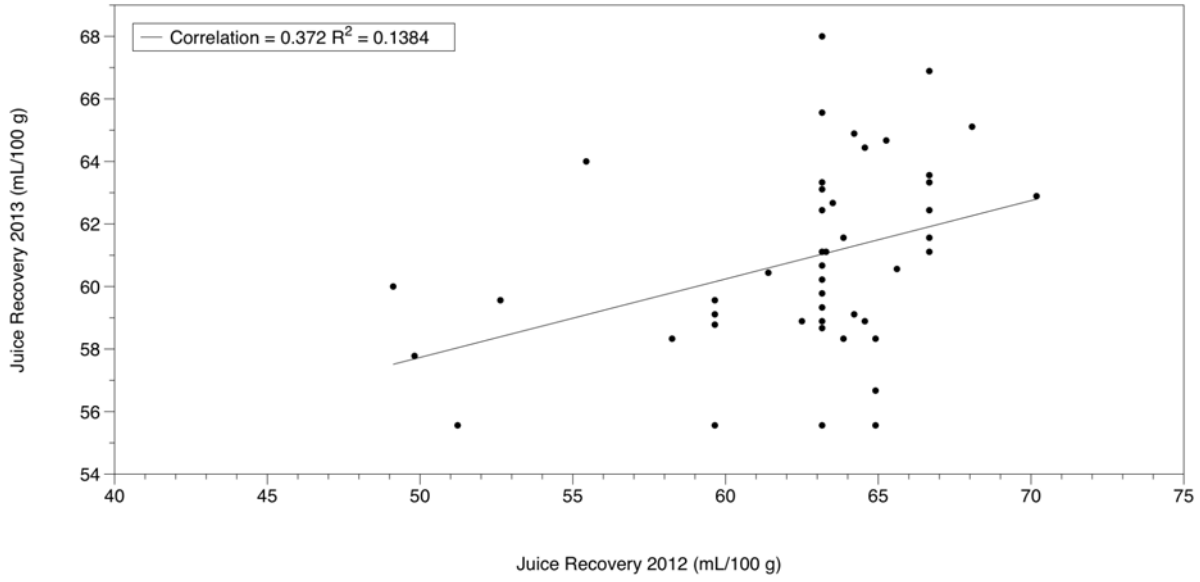


Figure 19. Comparison of juice extractability of 47 white selections for 2012 and 2013.



To assist in the selection of elite individuals, those selections that consistently exhibit high levels of berry shrivel over several seasons could be culled (discarded). Berry weight (size) does not appear to be a trait worth screening for in winegrape selections as berry size is a minor component of yield compared to bunch and berry number and there was only a weak non-significant correlation with juice extractability and berry weight.

4.4.4 Fruitfulness and yield

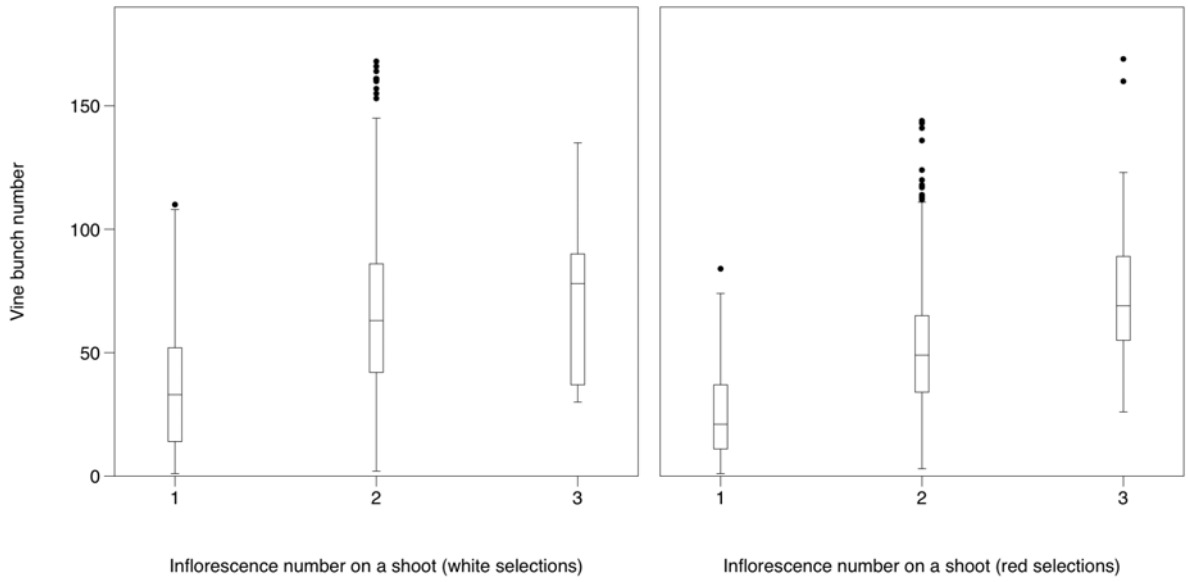
Grape yield per hectare is essential for profitability for both the viticulturist and winemaker and the number of vines per hectare will change yield. Yield per vine can be influenced by management (e.g., number and length of cordons, training, water application), environment, and genetics (variety) see Fig. 6 in (Carmona *et al*., 2008). The genetic control of yield is complex as floral induction, bunch development and berry development occurs over a 1.5 year period. The environmental conditions during the two Spring and Summer seasons over this period influence final yield with the first Spring and Summer affecting floral induction in the latent bud and the following Spring influencing inflorescence size and fruitset. To investigate if there was variation in fruitfulness and yield a number of traits involved in yield were scored.

A simplified yield model for a single vine is:
Bunches per vine x berries per bunch = yield (kg/vine)
Where:
Bunches per vine = bunches per shoot x total fruitful shoots per vine

Berries per bunch = flower number x percent fruitset

The number of inflorescences on a shoot was evaluated for the selections (Fig. 20). Selections were grouped on whether they had shoots with only 1 inflorescence, 2 inflorescences or 3 inflorescences. To simplify scoring and analysis, if for example, a selection had some shoots with 3 inflorescences and other shoots with 2 or 1 inflorescences the selection was scored as “3” as this represented the maximum potential of that vine. It can be seen that for both white and red selections there was a trend for higher total bunches per vine at harvest with increasing inflorescence number on a cane (Fig. 20). Vines scored as having 1 bunch on a cane had a median bunch count of 21-33 compared to selections having 3 inflorescences on a cane producing a medium bunch count at harvest of 69-78. Some vines produced no inflorescences and bunches and were excluded from the analysis.

Figure 20. Whisker box plot of selections grouped into inflorescence number on a shoot and bunch number at harvest. Data collected for 2013 for 583 red selections and 596 white selections.



Although data interpretation is limited because total vine shoot numbers with an inflorescence was not counted it appears that further study into the genetic control of inflorescence number on a shoot is warranted. A likely developmental stage to target for investigation would be floral induction in the latent bud as this determines the presence or absence of inflorescences post bud burst in the following year.

Total vine yield was correlated with bunch number and a significant association (R^2 0.79) was found for both red and white selections using a power function to fit a linear line (Fig. 21). In contrast there was no significant correlation between bunch size (average bunch weight) and total vine yield (Fig. 22).

Figure 21. Correlation of vine yield and bunch number. Colour coding represents individuals from different progeny populations. 2013 data for 596 white selections and 583 red selections.

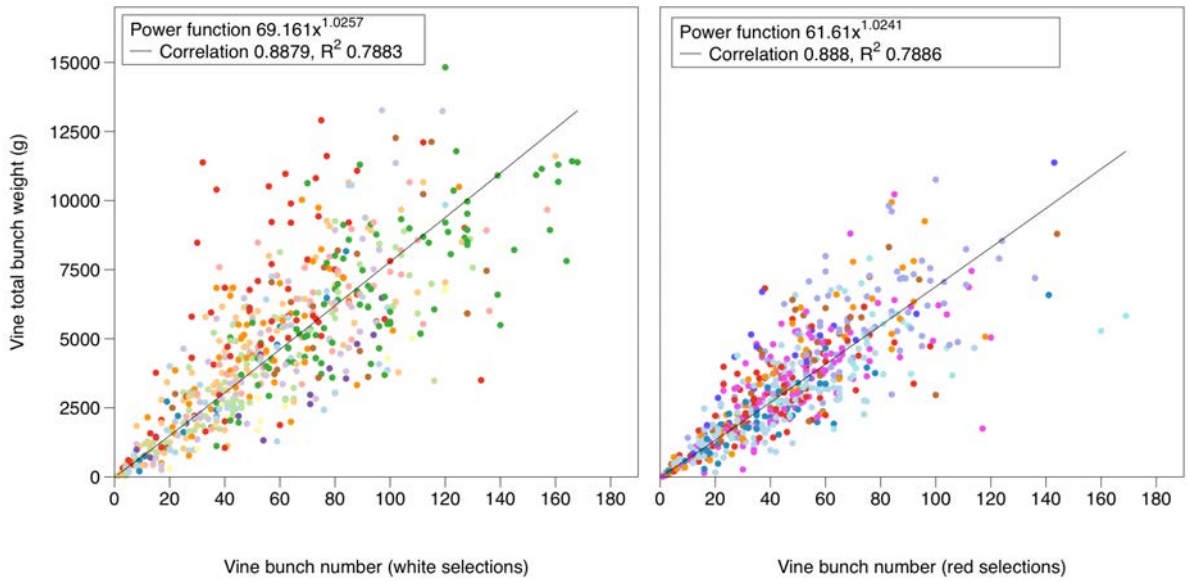
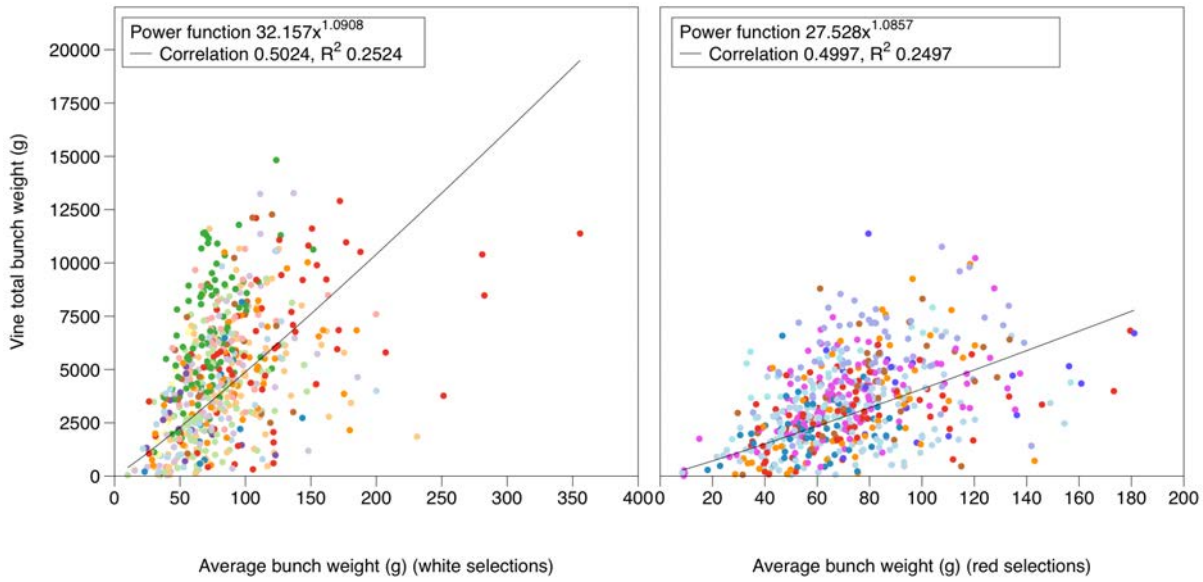


Figure 22. Correlation of vine yield and average bunch weight. Colour coding represents individuals from different progeny populations. 2013 data for 596 white selections and 583 red selections.



The results present in Figs. 21 and 22 confirm that vine bunch number is the major factor contributing to yield with the minor contributing component bunch weight.

Fruitset is a factor influencing berry number per bunch and final yield. Data collected for two years (2012, 2013) found 4 selections that had poor fruitset suggesting that there might be a genetic component determining this trait for these individuals.

Fruitful basal buds are important for vines that are spur pruned. To determine if there was variation for basal bud fruitfulness the selections were assessed for the presence of inflorescences on canes that had been spur pruned. It was found that over 2 seasons 99.55% of 1109 selections had fruitful basal nodes.

During the evaluation period no attempt was made to prune each selection to a set number of buds for the comparison of yield as the intention was to establish the vines and determine yield potential and wine attributes in the shortest possible time.

To assist in the selection of elite individuals, those selections that produce more bunches per vine should be targeted. The trend for selections with 2 or 3 inflorescences on each shoot to have a high number of bunches per vine suggests that the genetic basis of this trait might be of future research interest. Collection of data over several seasons might also allow a genetic analysis of the yield and vine bunch number between progeny populations to investigate the heritability of these traits and if some parents produce higher yielding progeny. Selections that consistently exhibit poor fruitset or no fruitful basal nodes over several seasons could be culled (discarded). The measurement of average bunch weight appears to be a poor predictor of total vine yield in this situation but a better correlation might be obtained if selections were pruned to have the same bud number.

4.4.5 Relationship between vigour and yield

Rapid vine establishment and growth is a valuable trait for reaching maximum fruit production as quickly as possible in the following seasons. There is a large body of literature on the effect of vine vigour and crop load (vine balance) on wine “quality” but these studies have focussed on traditional scion varieties (Bravdo *et al*., 1985; Jackson and Lombard, 1993; Howell, 2001; Kliewer and Dokoozlian, 2005; Terry and Kurtural, 2011). Rootstocks and management techniques that control water application and/or crop regulation (pruning, shoot thinning, bunch removal) are used in the wine and grape industry to modify vine balance and yield to achieve winery specifications. However, regional and seasonal differences appear to have a greater influence on yield than management techniques (Antcliff, 1965; Clingeleffer, 1984; Bramley and Hamilton, 2004; Keller *et al*., 2004; Clingeleffer, 2006).

To investigate if there was a relationship between yield potential and vine vigour an analysis was performed with yield data from the 2013 season. It was found that there was no significant correlation across the selections, however, a potential correlation was found within one progeny population (Figs. 23, 24). For cross C241 there was an improved correlation between trunk circumference and total vine yield (Fig. 23). This suggests that vine vigour, indirectly measured as trunk circumference, may have a relationship to final grape yield and one hypothesis is that both are related to carbohydrate production (photosynthesis), storage and utilisation. Selections that produce more sugar would be more vigorous and have a higher yield with yield also modified by other genetic factors controlling fruitfulness. Future studies aimed at the genetic dissection of carbohydrate production efficiency and yield may provide a basis for selecting elite plants.

Figure 23. Correlation of vine trunk circumference with either vine yield or bunch number. Colour coding represents individuals from different progeny populations. 2013 data for 596 white selections and 583 red selections.

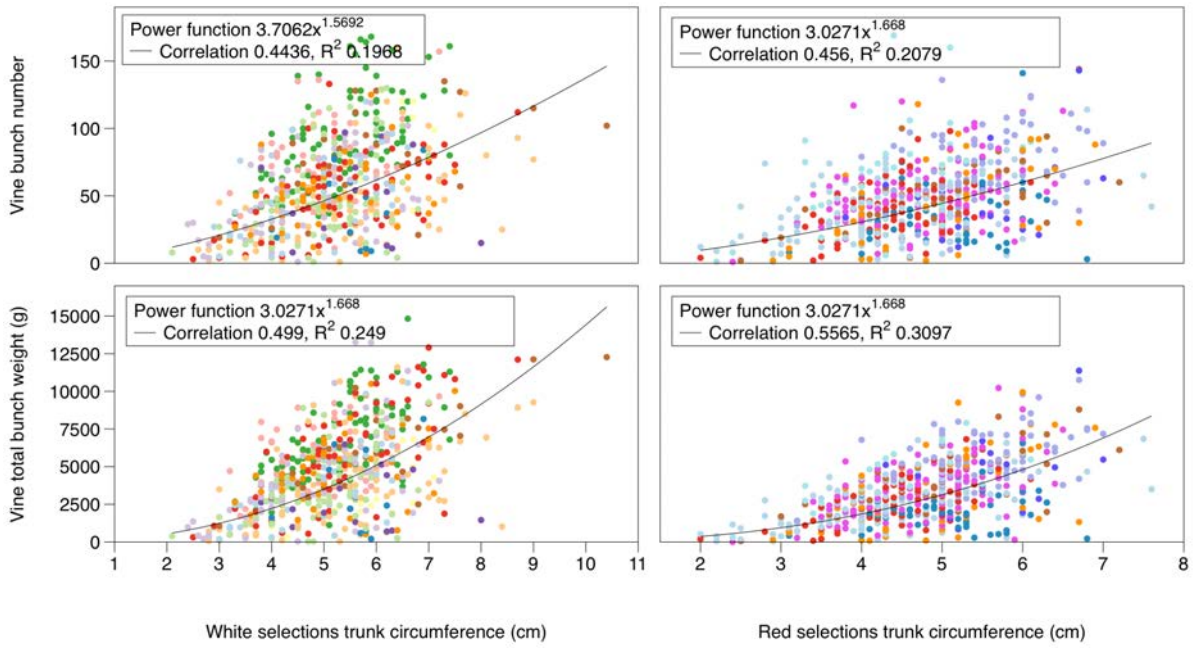
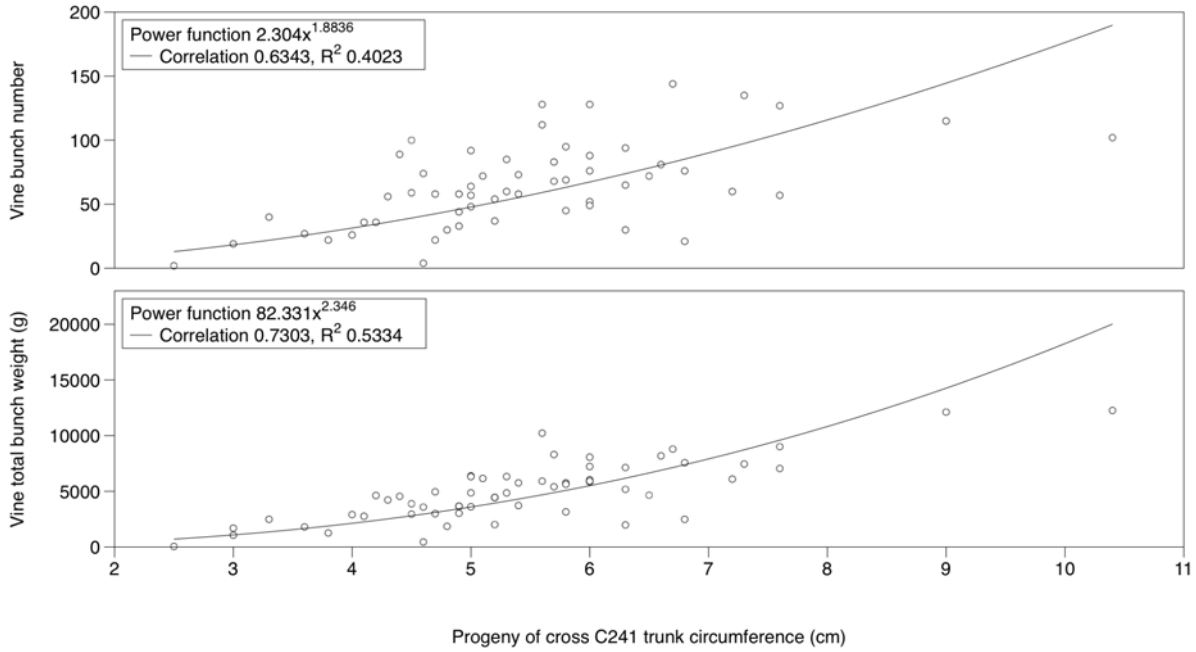


Figure 24. Correlation of vine trunk circumference with either vine yield or bunch number for individuals from cross C241. 2013 data.



4.4.6 Mildew and botrytis resistance in the field

The selections were planted in a no-spray vineyard and assessed yearly for the presence of downy mildew, powdery mildew and botrytis. No downy or powdery mildew was observed during the 3 years of growth in the field. The 2011 season in the Barossa Valley was a high disease pressure year due to the weather and the selections had no evidence of powdery or downy mildew on leaves or pre- and post-veraison fruit (Fig. 25).

The selections were not resistant to botrytis infection. During seasons that had wet periods during the maturation and harvest period the crop was damaged by botrytis. The 2011 season was particularly wet during the berry maturation and harvest period and it is suggested that the selections be sprayed to control botrytis in the future during years when the weather is conducive to botrytis outbreaks.

Figure 25. Mildew resistance in the field under no-spray management conditions. Pre-veraison control bunch from a susceptible *V. vinifera* variety (White Grenache) showing mildew infection (left). Pre-veraison bunches of a selection showing no damage or infection (right). Control plants and selections were within 100 m of each other.



4.4.7 Phenology

Anthesis date, the length of the pre-veraision period and the post-veraison rate of berry sugar accumulation will influence the date of berry maturity and harvest. These biological processes are very different and are likely under the control of different regulatory genetic pathways. To determine if there was variation between selections for these traits; anthesis date, veraison date and crop maturity dates were recorded with data collected on a weekly basis. Berry maturity, as determined by measuring total soluble solids as degrees Brix (°Bx), was used to determine the harvest date of the crop. The °Bx berry maturity value was 22 °Bx for white selections and 23 °Bx for red selections.

Both white and red selections had similar anthesis dates with flowering occurring over a 17 day period between 2–19 November 2012. The majority of the selections flowered within 7 days (6-12 November) of each other (Fig. 26).

Veraison date for whites was recorded as berry softening and berry colour change was used for red selections. It was found that veraison was spread over 27 days for white selections (9/1/2013 – 5/2/2013) and 47 days (27/12/2012 – 12/2/2013) for red selections (Fig. 27). There was greater variation in the veraison date for the selections compared to the anthesis date.

Harvest date and berry °Bx values at harvest showed that there were selections that matured early (6/2/2013) and those that either matured late (21/3/2013) or did not reach the harvest °Bx value on this date (Figs. 28, 29). The period of 43 days from the earliest harvest to the last harvest suggests that there is genetic variation within the selections for sugar accumulation during the berry post-veraison period. However, the observed large variation in the veraison date and smaller variation in anthesis date may also influence the harvest date with those selections that reach veraison early also having an early harvest date.

A preliminary study was carried out on yield and harvest date (Figs 30, 31). For the 2013 data presented the red selections were in their second cropping season and the white selections a mix of both second and third cropping season. Caution should be exercised with data interpretation as crop load was not standardised across the selections to allow investigating of the full potential of these selections to see if the selections could ripen large crop loads and no attempt was made to reduce bunch numbers per vine or bud numbers during winter pruning. Crop load is known to influence harvest date (Antcliff, 1965; Bravdo *et al*., 1985; Keller *et al*., 2004; Kliewer and Dokoozlian, 2005). Despite these limitations it can be seen that some of the early harvest white selections had high yields up to 10 kg and red selections up to 3.7 kg (Figs. 30, 31). The higher target 23 °Bx for red selections compared to 22 °Bx for white selections in addition to the younger age of the red selections is the likely cause of the smaller yields observed for the early harvest red selections. Many white and red selections achieved single vine yields above 5 kg and ripened before 14 March 2013. These are promising high yielding selections for further study.

Figure 26. Anthesis variation dates in November 2012 for the 2013 crop. Data for 599 white selections and 587 red selections.

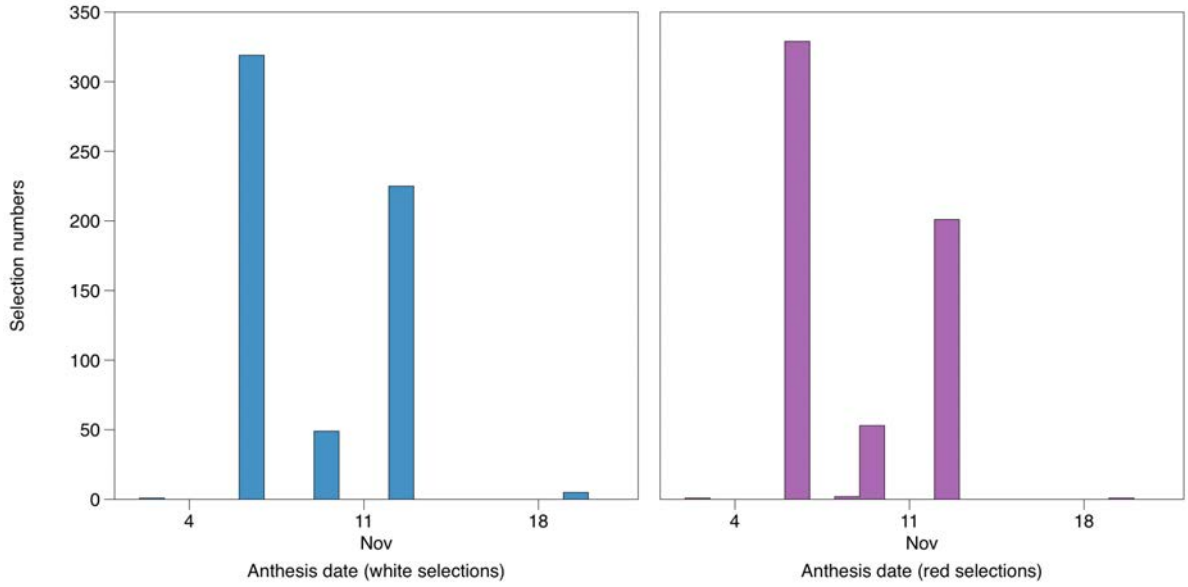


Figure 27. Veraison dates for the 2013 crop.

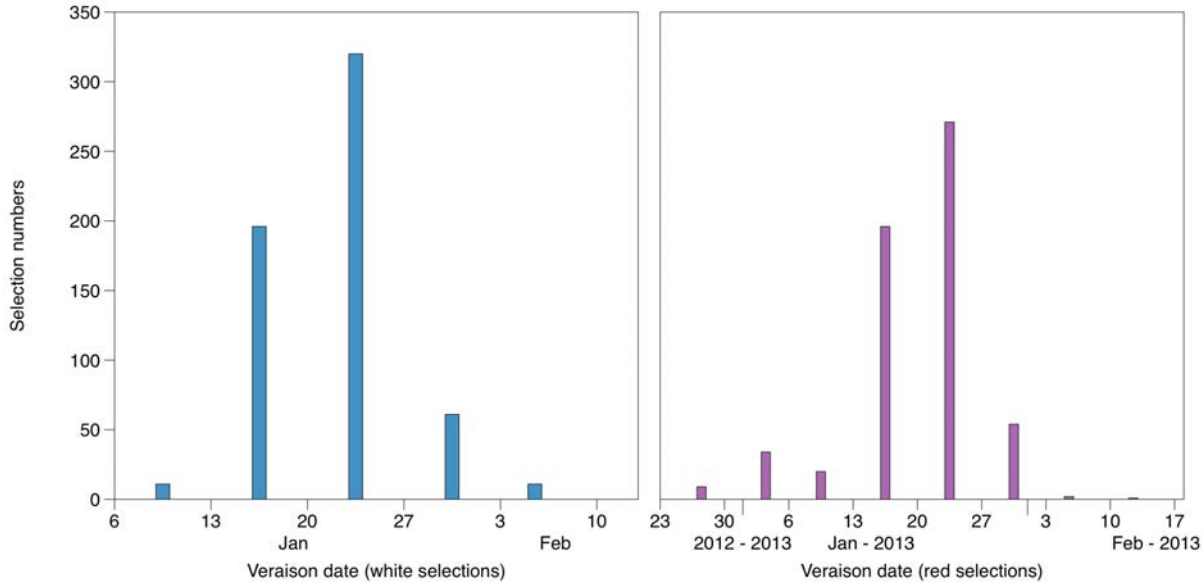


Figure 28. Harvest dates for the 2013 crop.

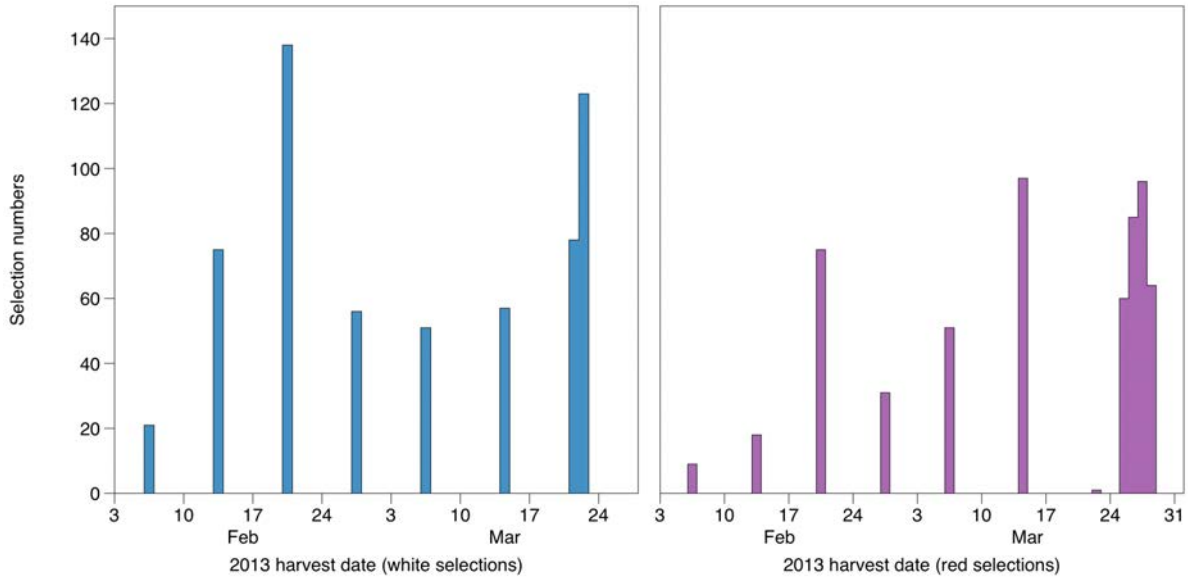


Figure 29. Degree brix values and harvest dates for the 2013 crop. Histograms at each date indicate the number of selections with a specific °Bx value. The data for the 21/03/13 represents all harvests at and after this date (see Fig. 28 for harvest dates).

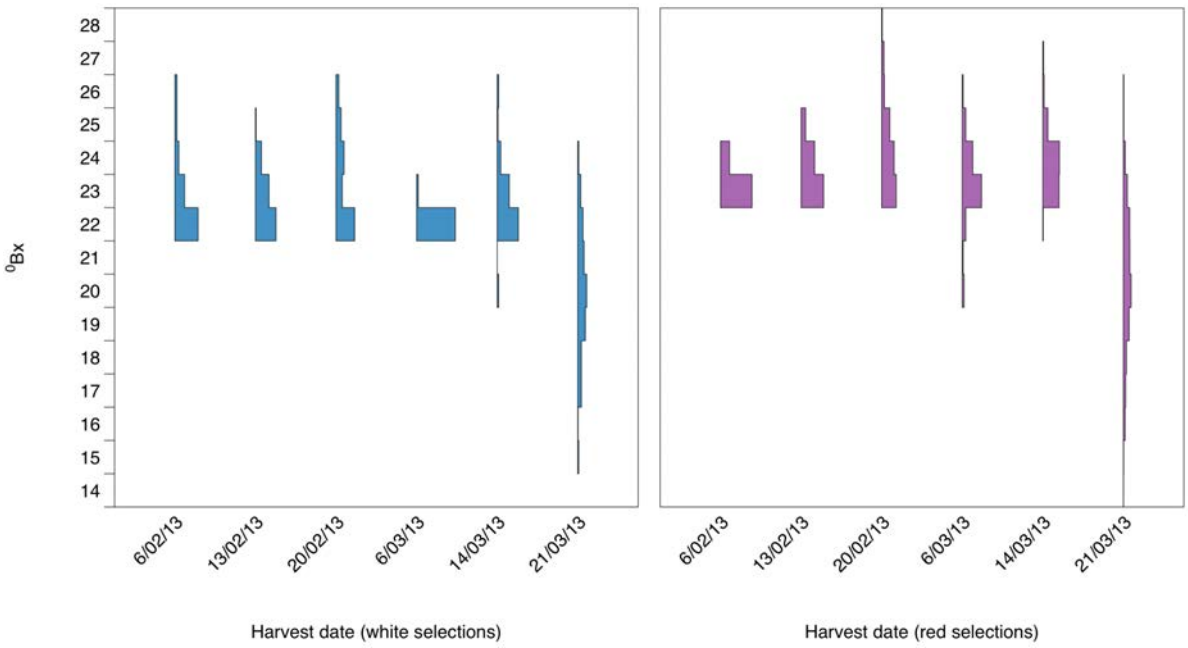


Figure 30. Yield and harvest dates for white selections. Data for the 2013 crop.

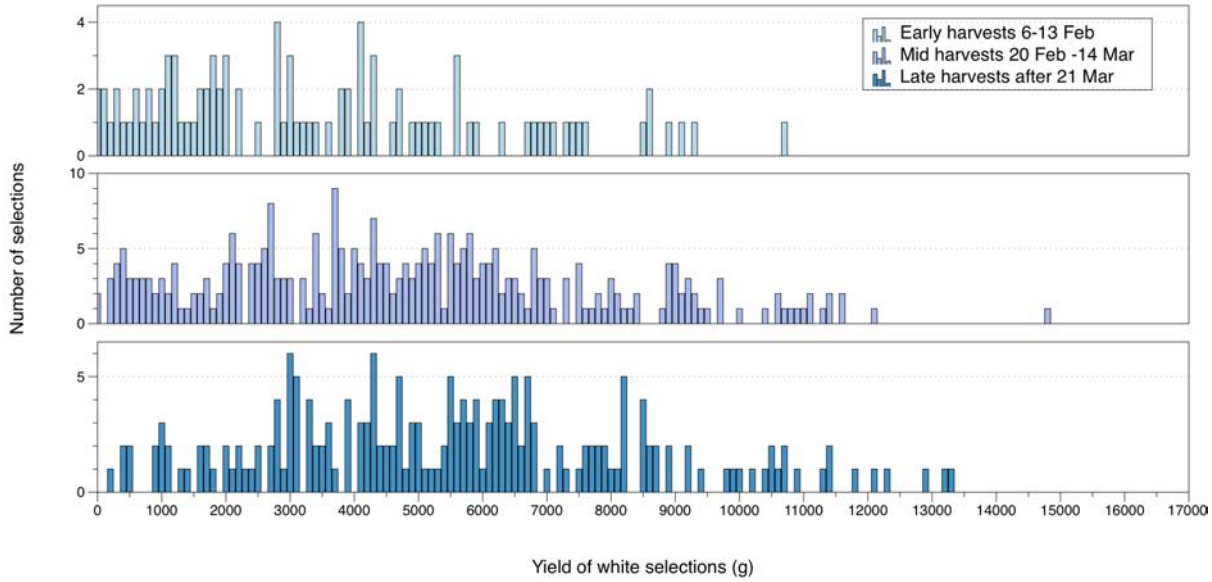


Figure 31. Yield and harvest dates for red selections. Data for the 2013 crop.

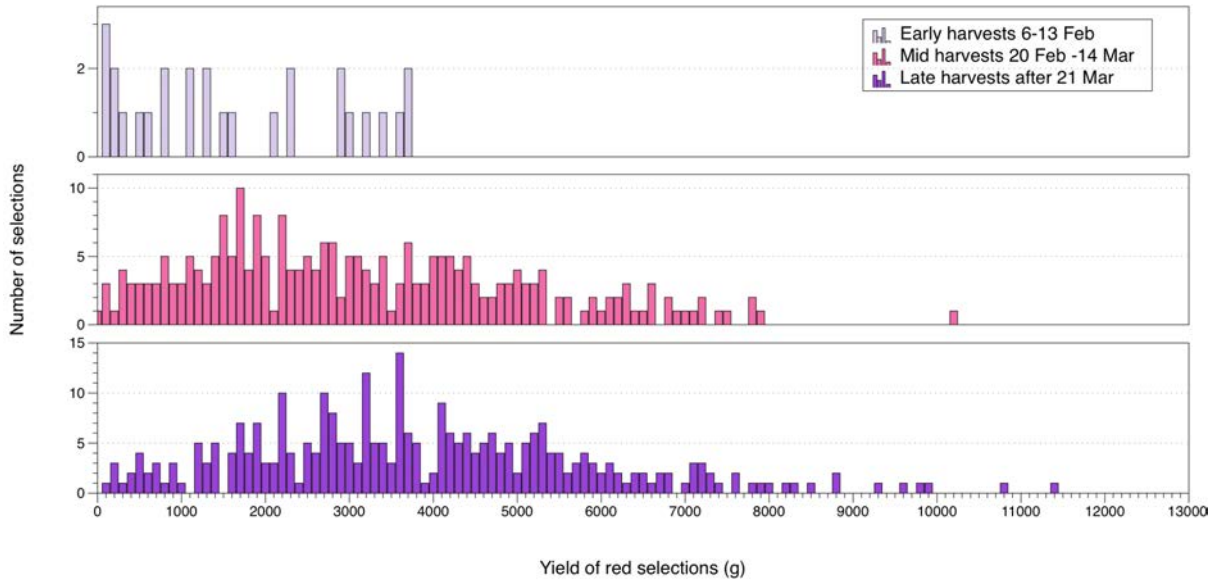
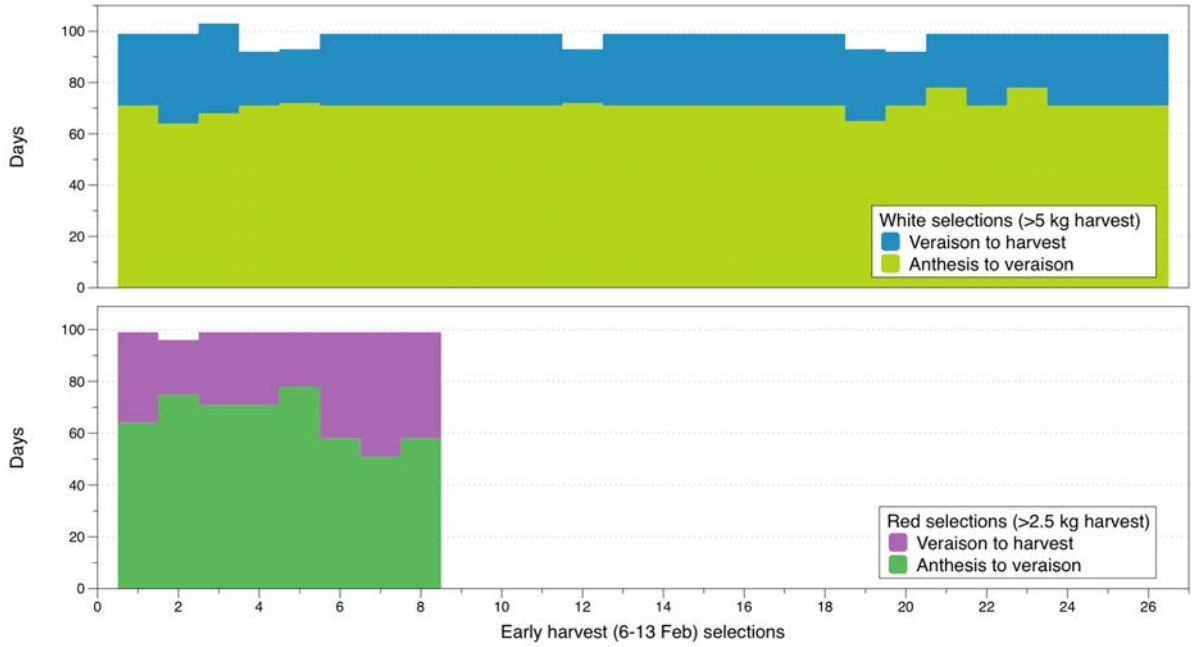


Figure 32. Period from anthesis to harvest for early maturing selections.



Selections that produced fruit that matured early were looked at to see if there was variation in the pre-veraison and post-veraison periods (Fig. 32). To reduce variability due to crop yield only white selections producing more than 5 kg or red selections producing more than 2.5 kg were investigated. The total period from anthesis to harvest was in the majority of cases close to 100 days, however, some had a reduced total period of 90 days due either to a shorter post-verasion period or in one case a shorter pre-veraison period (Fig. 32).

To assist in the selection of elite individuals, those selections that produce high yields at early maturity dates should be targeted in future studies. Late maturing selections and those that did not reach the target maturity °Bx value could be culled (discarded) as these would be of no interest to industry. Selections that have high yields and reach maturity early would be of benefit to industry as they would require less resources (water, management) from budburst to harvest and would avoid damaging weather events (heat stress, hail & rain damage) late in the season.

4.5 Grape and wine analysis

Rapid high throughput methods to measure small quantities of grapes and make small volumes of wine are required to screen large numbers of selections to determine the variation within the selections and assist in the identification of elite selections. This section describes progress in achieving this aim.

4.5.1 Micro-scale winemaking

Bunches of grapes from selected white selections were stored in a freezer until processed for winemaking. This allowed selections that were harvested on different dates to be processed for winemaking in the same batch. For white winemaking either 160 ml or 500 ml fermentations were developed and evaluated (see methods section & Fig. 33a,b). The 160 ml ferments were carried out for the 2011 and 2012 vintages and allowed up to 50 fermentations in a single batch. The 160 ml ferments on average were for 16 days with all wines in a batch racked on the same day. These small ferments were successfully used for both chemical and sensory analysis but the small final volume of wine restricted the number of people in the sensory panel. Issues were also experienced with storage of these small wine volumes prior to sensory evaluation as they were not filtered and the cap closure sometimes did not properly provide an airtight seal. For the 2013 vintage a new method was developed using frozen grapes that involved crushing 900 g of grapes and ferments of 500 ml. These fermentations lasted from 9 to 17 days and daily monitoring after 5 days ensured that the wine was racked when sugar levels were <2.5 g/L. The 500 ml fermentation volume allowed up to 29 fermentations to be processed in a single batch and provided a suitable volume of wine for sensory evaluation.

For red selections, a red winemaking method previously described for Pinot noir (Dambergs and Sparrow, 2010) was used with modification for the 2013 season. Freshly harvested grapes were collected and 1.3 kg crushed and fermented (see methods section & Fig. 33c). Ferments were from 4 to 8 days with wines racked when sugar levels were <2.5 g/L. Up to 35 fermentations could be processed in a single batch using this method. Fresh grapes were used instead of frozen grapes as a freeze thaw cycle would affect the extractability of colour and other compounds from the skin. No malolactic fermentation was done for the red wines due to the small fermentation volumes. The developed method produced wine suitable for both chemical and sensory analysis.

Figure 33. Micro-scale ferments; 160 ml white ferments (a), 500 ml white ferments (b) and 1.3 kg red ferments (c).



4.5.2 Chemical analysis

The purchase of an OenoFoss machine allowed the rapid measurement of a number of parameters for both berry and wine for the 2012 and 2013 seasons. Berry must results for both white and red selections show that there was a wide range in must pH level, °Bx, acid levels and YAN (Figs. 34, 35). Overall must °Bx values were lower than those measured in the field with a hand held Brix refractometer.

Figure 34. Variation in harvested berries from white selections for °Bx, pH, total acid to pH 7.0, tartaric acid, malic acid and YAN measured using an OenoFoss, Data from 582 selection in 2013.

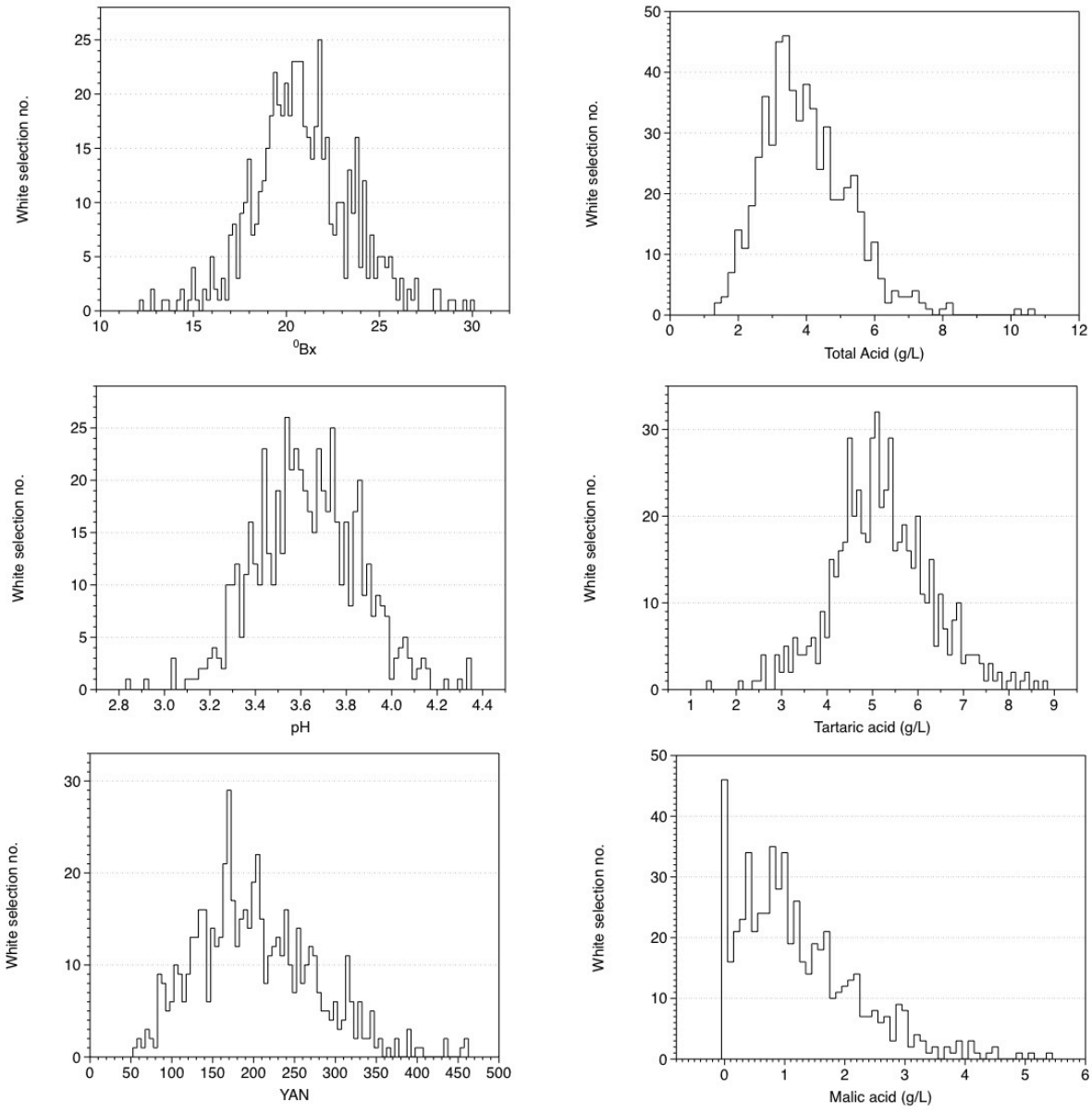
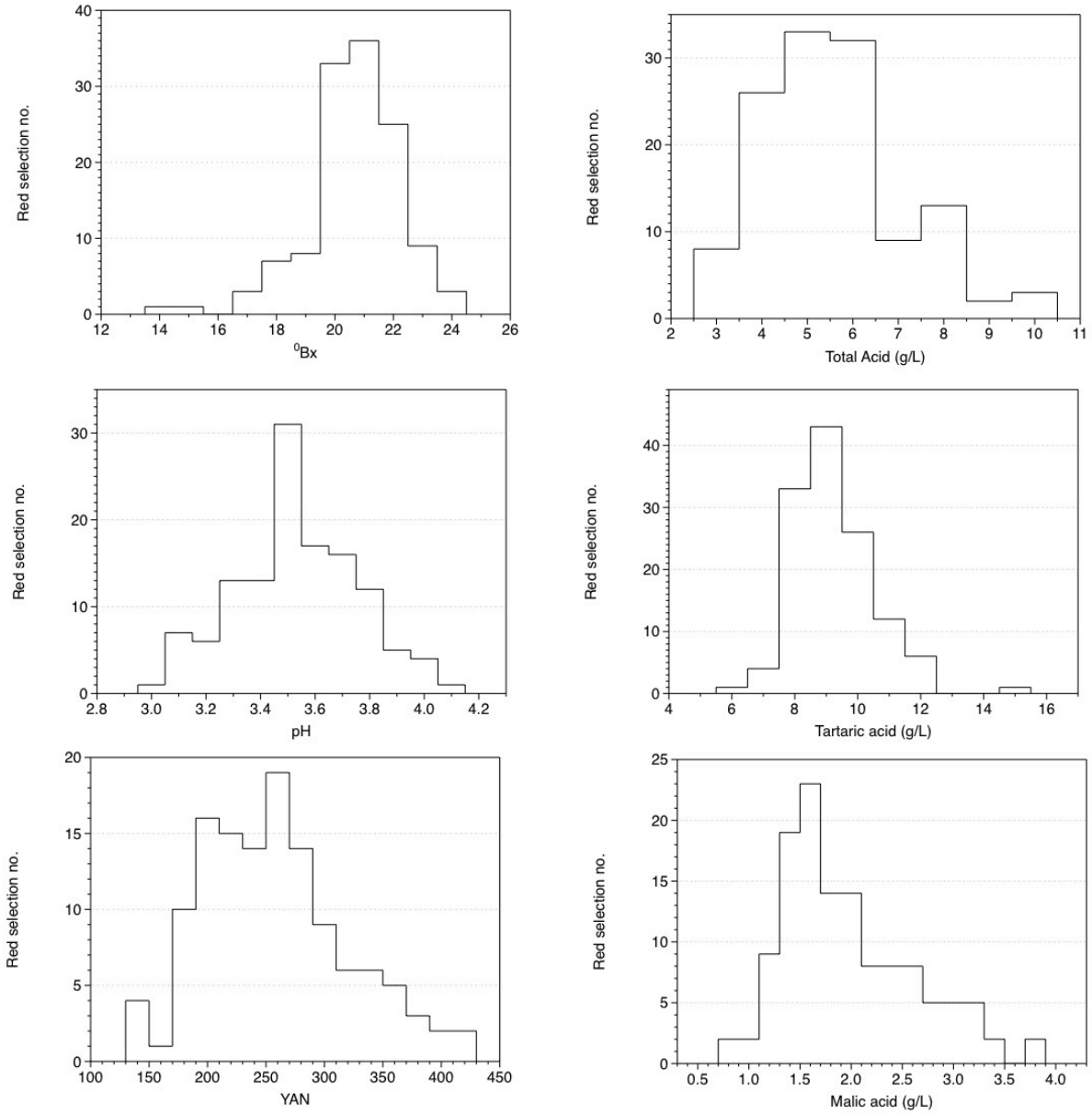


Figure 35. Variation in harvested berries from red selections for °Bx, pH, total acid to pH 7.0, tartaric acid, malic acid and YAN measured using an OenoFoss, Data from 128 selection in 2013.



Must pH values ranged from 2.97 – 4.0 for red selections with an average of pH 3.5 (Fig. 35). The white selections had an average must pH value of 3.6 with a range from 2.84 - 4.35 (Fig. 34). The ratio and levels of tartaric acid and malic acid in the berries determines pH and these levels vary within the selections (Figs. 34, 35). Acid addition to reduce the pH during winemaking can be a significant cost to a winery if the pH of must is high. It can be seen that there were a proportion of both red and white selections that had pH levels of 3.4 or below for the 2013 season (Fig. 34, 35) and these would not require acid addition during winemaking if the target pH was 3.4 or below.

Yeast Assimilable Nitrogen (YAN) varied significantly amongst the selections and Diammonium Phosphate was added for all ferments (see methods section) to prevent stuck ferments.

Chemical analysis was done on wine using the OenoFoss to measure, ethanol (% vol.), pH, TA to pH 7.0, malic acid, glucose + fructose, density and volatile acid. Glucose + fructose values confirmed the completion of fermentation. During the winemaking, adjustments were made to pH with tartartic acid to keep the pH at 3.4 or below. The volatile acid values for the wines were below 0.35 making them suitable for sensory analysis. The ethanol values for reds and wines are presented (Fig. 36). Some wines were deliberately made at low alcohol levels (Fig. 36) from early harvest material as a preliminary investigation into the suitability of some selections to produce low alcohol wines with flavour without any winery manipulations.

To investigate if there was a relationship across the two seasons 2012 and 2013 for the selections a comparison was carried out for pH, TA, malic acid and tartaric acid. It was found that there was a correlation between seasons for the different white selections for pH and TA (Fig. 37). Low correlations were found for malic and tartaric acid.

To assist in the selection of elite individuals, those selections that have pH values below 3.5 at harvest consistently over a number of seasons may be attractive to industry as they would require little or no acid addition in the winery.

Figure 36. Ethanol levels of wines made for sensory analysis. 2013 season.

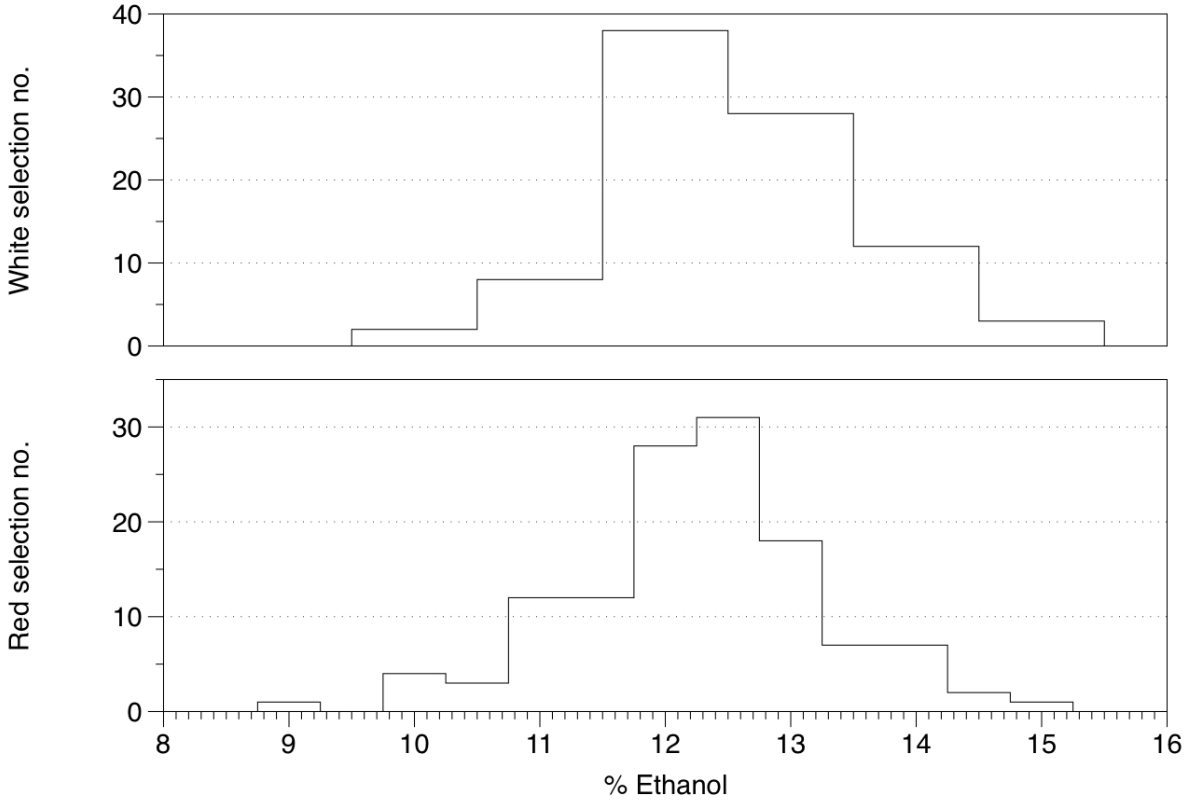
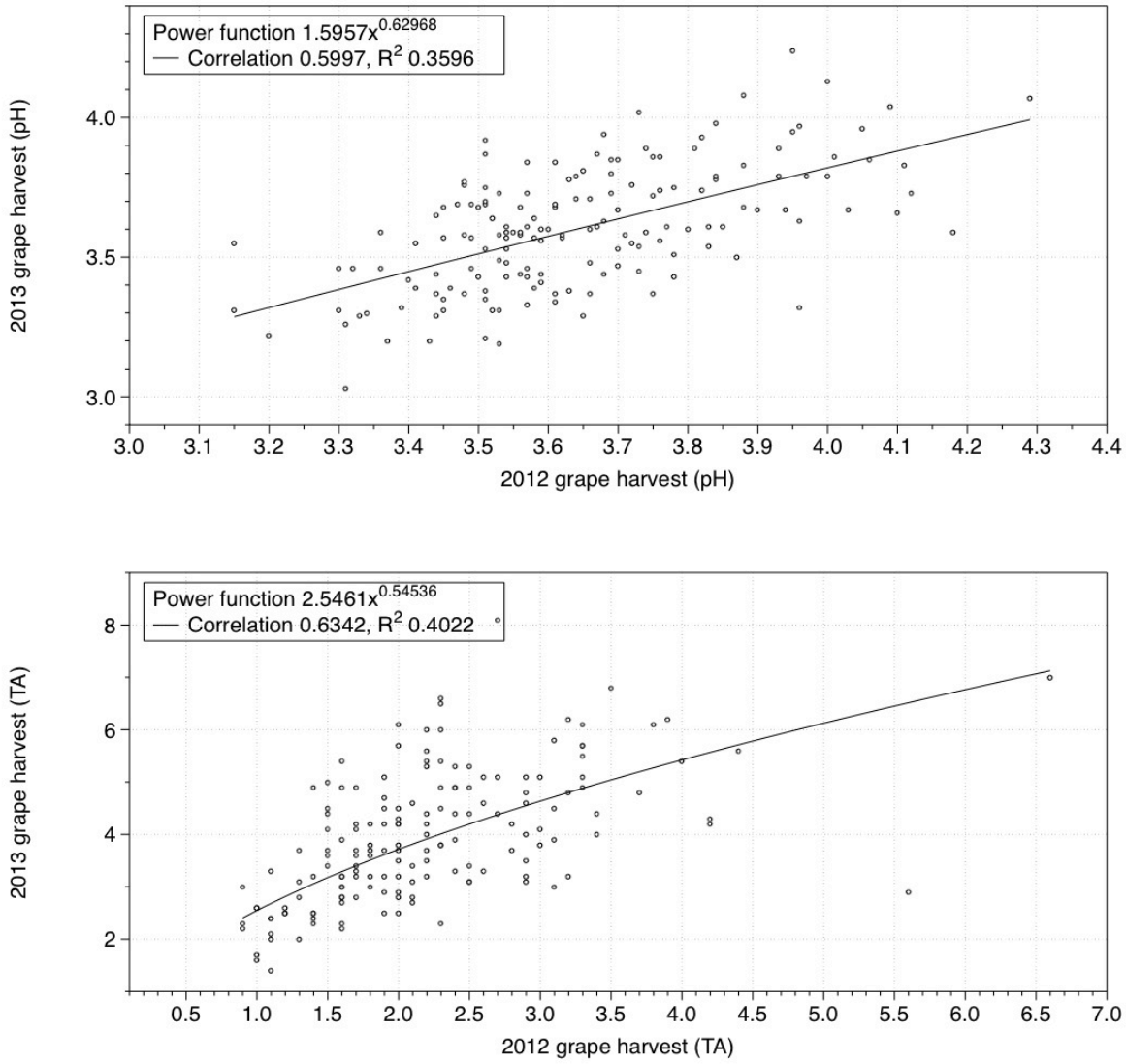


Figure 37. Correlation across 2012 and 2013 seasons for white selections measured for pH and TA (total acid to pH 7.0). Data for 161 white selections.



4.5.3 Sensory evaluation

Sensory analysis of 160 ml white wine ferments was carried out for the 2011 and 2012. Sensory analysis of the 2013 white and red selections was carried out after the completion of this project and is not reported here. The sensory analysis for 2011 and 2012 wines was scored by small group of 5 commercial winemakers. The wines were scored for a number attributes in an attempt to group the wines on dominant flavour profile and commercial interest level (Fig. 38). There was a wide range of flavour types identified by the sensory panel (Fig. 39). One of the interesting findings was the large number of selections identified as having a unique flavour (Fig. 39). The interest level was averaged across the winemakers and it was found that some selections ranked highly across multiple winemakers with the control Chardonnay (tasted blind) scoring zero interest (Fig. 40).

Figure 38. Example of a scorecard for a white wine (selection 39_16_03_01) scored by a commercial winemaker.

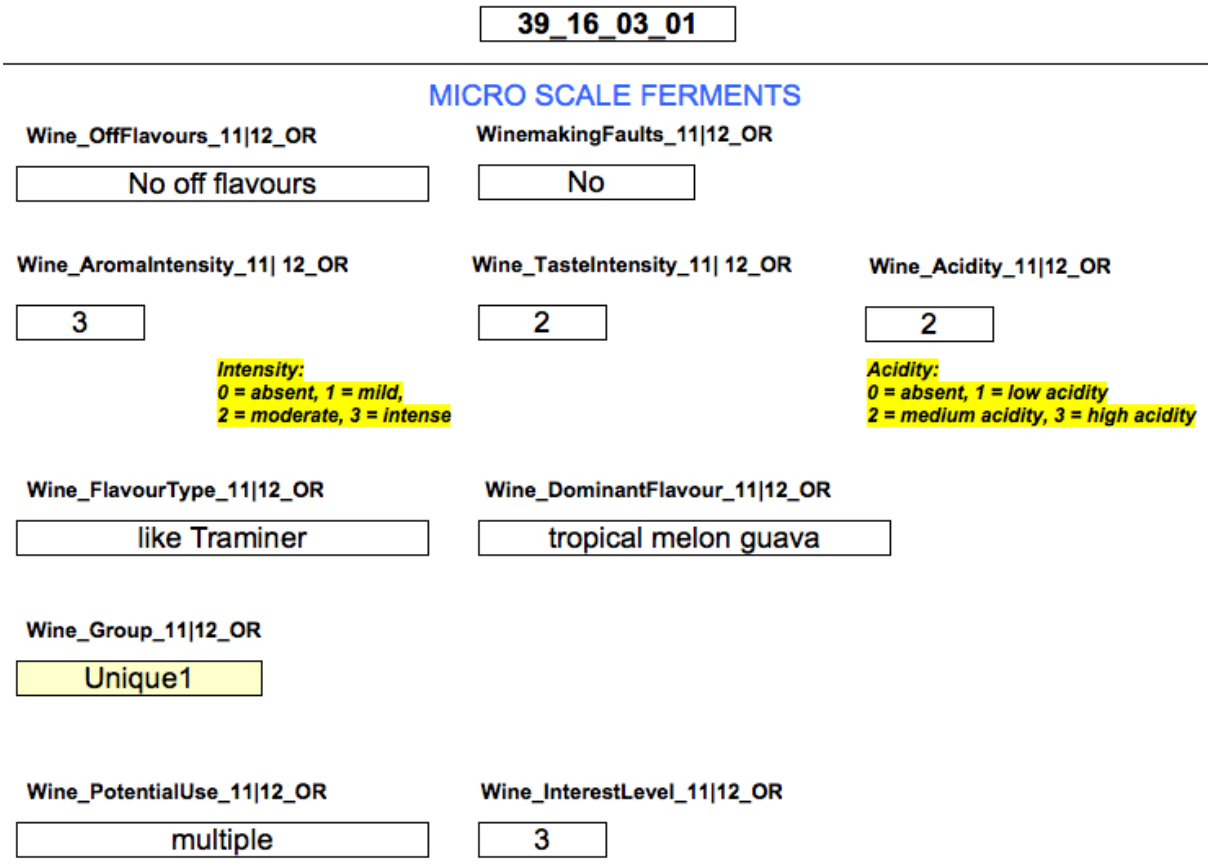
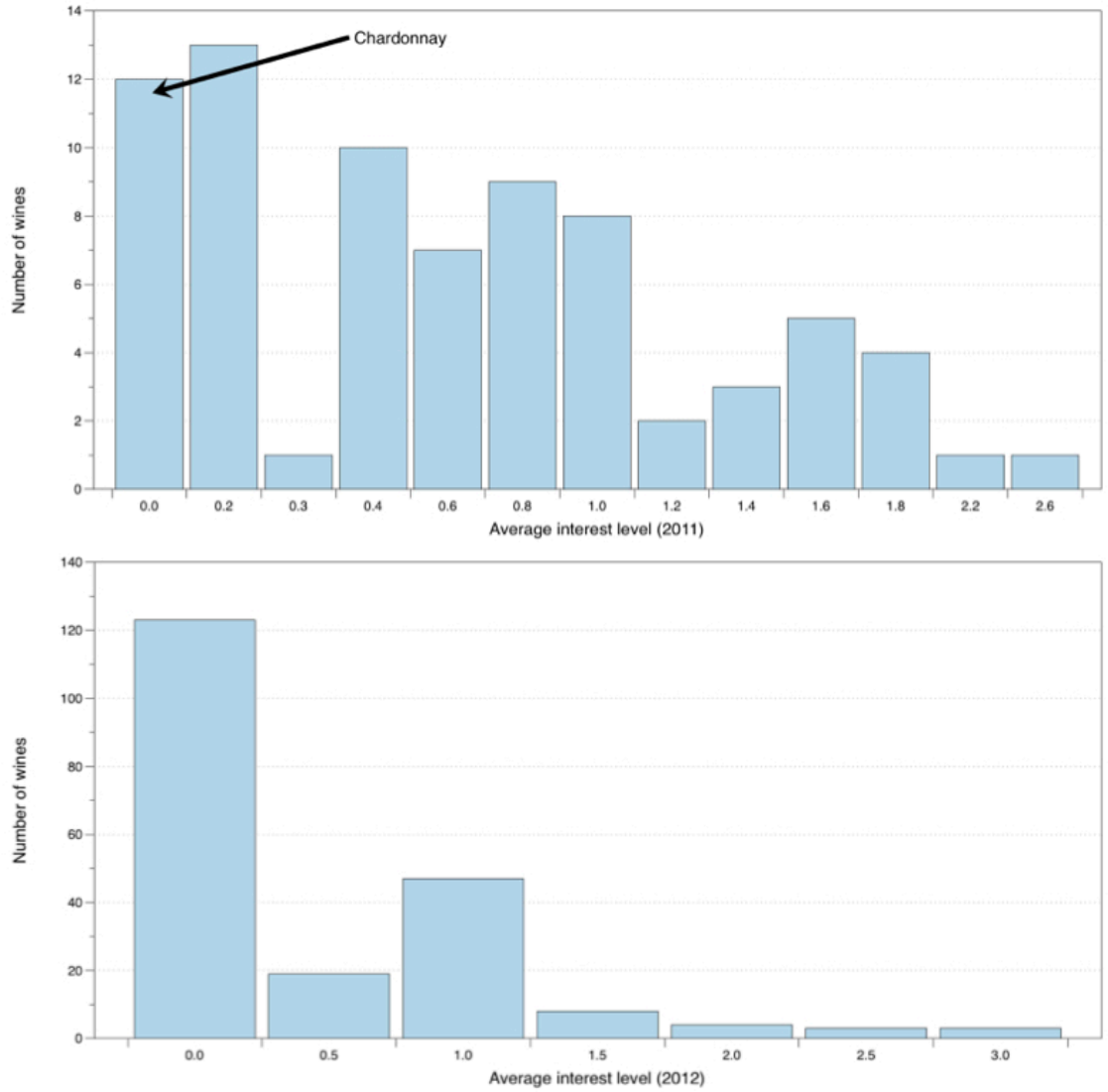


Figure 39. Word cloud for white wines scored for flavour type. Data from 2011 (a) and 2012 (b). The larger the text the higher the number of wines in that group.

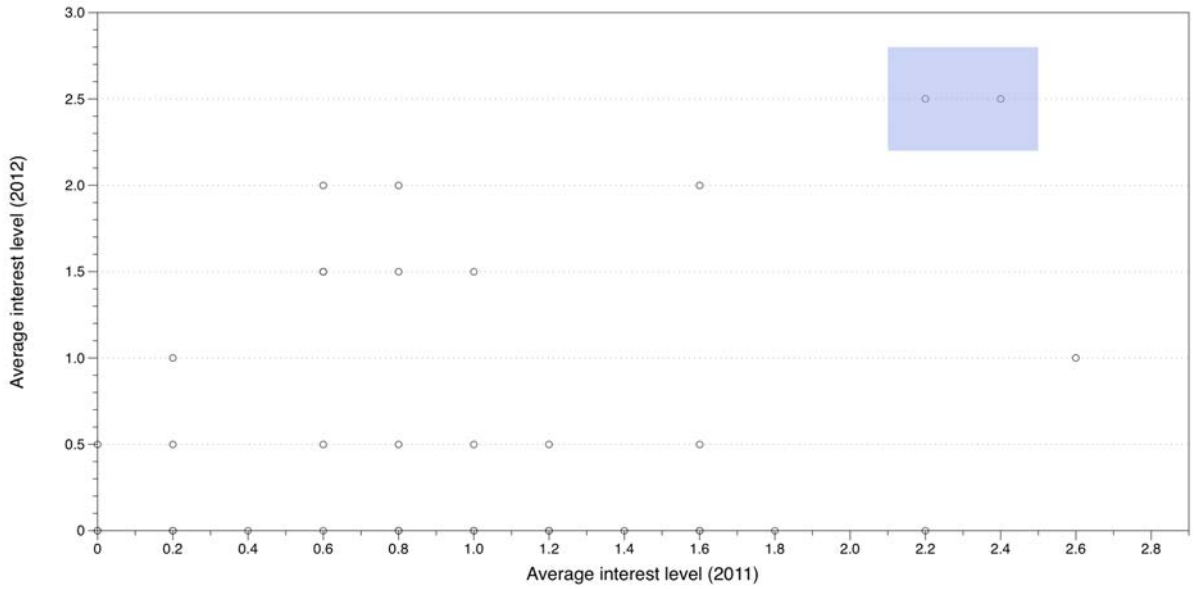


Figure 40. Average interest level for white wines made in 2011 (top) and 2012 (bottom). 2011 data from 5 winemakers for 76 selections including Chardonnay as a control. 2012 data from 4 winemakers for 170 selections. Interest level was scored from 0 (no interest) to 3 (high interest).



To investigate consistency of scoring across years, selections that had wine produced from both years were investigated. It was found that there was little consistency apart from two selections (Fig. 41). This low consistency was to be expected due to the variables of micro-scale winemaking and subjective scoring carried out in different years.

Figure 41. Average interest level for white wines made in 2011 and 2012. Highlighted selections were scored as being of high interest in both years.



A preliminary investigation was made into the potential for red flesh selections to produce low alcohol red wines by harvesting early at a low °Bx level. The wines were made using the white wine method with no skin contact. The wines were sampled informally by the winemakers and described as like a beaujolais style with flavour. The selections produced reasonable wine colour levels (Fig. 42) when harvested at low °Bx levels and made with no skin contact suggesting that some of these selections with further investigation might be suitable for dry lighter alcohol wines without winery manipulations if harvested early. A style developed in this way would also spread the harvest period and may be seen as of benefit for some wineries.

Figure 42. Low alcohol red wines produced with no skin contact from early harvest red flesh selections. 9.5% (left), 10% (centre) and 12% ethanol (right).



Sensory evaluation of wines by commercial winemakers proved to be useful in classifying selections into groups with similar flavour. The use of winemakers to classify wines into those of low and high interest was a useful exercise but the subjective nature of the assessment combined with the different preferences of the winemakers and the diverse wine flavours made interpretation of the data difficult for identifying selections of high commercial interest. Further research into rapid methods for classifying selections into flavour groups and identifying those of high industry interest is required.

4.6 Phenotyping selections in the vineyard and winery

Sections 4.4 and 4.5 described the various traits and measurements on vines, grapes and wines used to evaluate the selections planted in the field. None of the methods could be described as high throughput making the screening of large numbers of plants to identify elite plants difficult. This becomes especially difficult when the selections have a diverse flavour type from ‘chardonnay’ to unique. In addition it is not possible to predict what might be a future popular wine style or what style one wine company may prefer to develop and market compared to another company.

However, the information presented in this report does suggest a way forward for developing better strategies for rapidly screening large numbers of field planted selections to cull inferior plants to identify elite plants. Selections to cull would be identified as having:
- low vigour
- low yield
- tight (compact) bunches
- high levels of berry shrivel
- berries that do not reach maturity by the last harvest date.

Selections that remain after the above cull criteria were applied would then be classified into flavour types based on either berry or wine sensory analysis using a trained sensory panel. The wines within each group could then be assessed and ranked by a trained panel or commercial winemaker panel. This would require winemaking volumes larger than 160 ml to produce enough wine for the different panels. In anticipation of this, larger scale ferments of 500 ml (white selections) and 1.3 kg (red selections) were trialled in 2013.

The approach would reduce the number of vines requiring berry and wine assessment. An additional screen for berry pH at harvest could also assist in culling selections that require the addition of tartaric acid in the winery.

The large seasonal fluctuations in weather require plants to be assessed over a number of years, however, one solution to reduce the time required would be to replicate the selections in different regions so that data could be collected from multiple environments in a single year.

Many of the white and red selections achieved single vine yields above 5 kg and ripened before 14 March in 2013. These are promising high yielding selections for further study. However the yield data by design had a degree of “noise” as the selections were not pruned to the same number of buds. This was done deliberately in these initial years after planting to assess vigour and observe the potential of the selections to produce and ripen large crop loads. In future years the option exists for elite selections remaining after culling to be pruned to a set bud number to observe the effect on yield and wine attributes.

A number of interesting results warrant further study that may eventually lead to rapid screens. The trend for selections with 2 or 3 inflorescences on a shoot to have a high number of bunches per vine suggests that genetic dissection of this trait might eventually lead to a DNA marker for fruitfulness. Vine vigour (measured as trunk circumference) may have a relationship to final grape yield and leaf greenness. Carbohydrate production (photosynthesis), storage and utilisation are key to vine productivity. Selections that produce more sugar would be expected to be more

vigorous and have a higher yield with yield also modified by other genetic factors controlling fruitfulness. Future studies aimed at the genetic dissection of carbohydrate production efficiency and yield may provide a basis for selecting elite plants. The ability of some selections to maintain a healthy dark green leaf colour up to harvest may be indicative of efficient nutrient acquisition and greater potential for carbohydrate production and this was observed when leaf greenness was related to vigour. There are stay-green (maintain a dark green leaf colour) varieties in other crops that have increased productivity (Hortensteiner, 2009).

5 Outcome & Conclusion

This project investigated the use of advanced genetic approaches for strategic and applied grapevine research.

A mutagenized microvine population was successfully developed for strategic research purposes using a novel mutagenesis method. This is the first report of successful mutagenesis by EMS of grapevine with both phenotypic and DNA evidence of mutagenesis. The development and use of a highly homozygous microvine line contributed to the success of the study. The rapid flowering phenotype of the microvine allowed M1 and M2 generations to be produced quickly and the homozygous line had the advantage of aiding in the identification DNA mutation sites in the genome.

The purpose of producing a mutagenized population was to assist future studies linking traits to genes. Linking traits to genes and DNA markers can also be achieved by studying the genetic segregation of traits in populations produced by controlled crossing of known parents. Studies were undertaken to identify genes or DNA markers linked to the fleshless berry locus, flower sex, mildew resistance, vine stature and berry colour. SNP DNA markers were successfully linked to these traits using microvine populations to assist in the rapid phenotyping of flower and berry traits. The mutated gene responsible for the fleshless berry phenotype was identified and a candidate gene controlling flower sex was also identified.

To investigate if SNP DNA markers could be used for marker-assisted selection (MAS) studies in grapevine a SNP DNA panel was developed and used for screening seedlings for berry colour, flower sex, mildew resistance and vine stature.

The SNP panel was successfully applied to screen over 8,000 seedlings with over 1,200 identified for further evaluation in the field. New methods were developed to achieve this result. It was found that the DNA panel accurately predicted the phenotype of the plant for the traits screened. This is a significant advance on current grapevine programs where vines are planted in the field and a period of years elapse before flower and berry traits can be scored. As an example, in this case over 8,000 plants would need to be planted in the field for evaluation if a traditional breeding program was undertaken but with MAS most could be culled in a glasshouse at the seedling stage as lacking one of the desirable traits and only 1,200 taken forward for field evaluation.

The selections were planted in a no-spray vineyard in the Barossa Valley as single vines (617 white selections and 627 red selections) and evaluation of agronomic

traits and berry and wine attributes was done. The vines showed no evidence of downy or powdery mildew in the field on leaves or berries but were not resistant to botrytis. It was found that there was large variation for vine vigour, yield, fruitfulness (inflorescence number on a cane), bunch architecture, bunch size, berry size and berry maturity (harvest) date. Chemical analysis of berries was carried out on those that produced fruit during the evaluation period and variation was found for °Bx, pH, TA, tartaric acid, malic acid and YAN. The measurement of berry pH at harvest may be useful in a future screen to identify elite selections that do not need the expensive addition of tartaric acid in the winery. The sensory evaluation of micro-scale white wines by commercial winemakers identified a wide range of flavour profiles in the selections with some selections being scored as of high interest by some of the winemakers. Section 4.6 describes in greater detail what strategies might be employed to assist in the rapid identification of elite selections.

6 Recommendations

6.1 Future Research Directions

The results reported conclusively demonstrate that strategic research aimed at linking DNA markers and genes to traits can be used in new breeding strategies such as marker assisted selection (MAS) to generate new grapevine varieties of potential benefit to the Australian industry.

Further research investigating genes controlling other key traits may produce new markers that can be incorporated into the SNP panel developed in this project to expand the number of traits that can be screened at the seedling stage. Traits that could be targeted include genes controlling berry traits such as muscat flavour, methoxypyrazines, bunch architecture and new sources of powdery and downy mildew resistance.

Based on the success reported in this project using MAS it is not difficult to imagine in the near future that the number of traits that can be screened using MAS will exceed what is feasible to physically screen in a glasshouse at the seedling stage. For example, if two parents were crossed and each parent had 10 traits of interest independently segregating on each of the 19 chromosomes it would require the DNA screening of 1,048,576 seedlings to identify 1 individual that combined all 20 traits. To overcome this problem a solution has been proposed that involves trait stacking in the microvine due to its ability to rapidly flower (Chaib *et al*., 2010).

This first generation of mildew resistant vines produced by MAS also provides a unique opportunity to investigate in detail the variation within this population for key agronomic and berry traits. Specific populations segregating for bunch openness

exist within the planting and there is also variation for organic acids. These progeny populations could be used to study the genetics of these traits to link DNA markers.

Further research is required to characterise in greater detail the wine attributes of the selections. Wines from red selections were only made from the 2013 vintage and sensory analysis remains to be done on these and white selections from the same vintage. The evaluation of traits especially those influenced by the environment require assessment over several years. This would require winemaking over several seasons and assessment of selected berry and agronomic traits

The identification of elite selections would provide both elite material for future breeding and material for industry adoption. Initial results have identified selections that produce high yields and mature early in the season as well as selections that produce wine of high interest to commercial winemakers but additional research is required to improve the selection process.

An ideal strategy to improve the efficiency of screening of selections in the field would be to evaluate the same selections in different regions similar to what occurs for cereal selections. In a single year, data for different environments would be obtained for the same selection instead of collecting data over a number of years at a single site. While this may not be possible for large numbers of grapevine selections it might be possible for groups of selected elites if the numbers were kept low.

6.2 Industry Context and Adoption Scenarios

Key drivers of profitability for the Australian wine industry are low production costs, high yields per hectare and a desirable marketable product. Improved planting material that reduce costs, produce new or improved wine styles and are better adapted to the changing Australian climate will provide industry with new options for maintaining profitability.

It has been demonstrated that the selections produced in this project do not require sprays for powdery and downy mildew in the Barossa region and this represents considerable cost savings to growers not only in chemical costs but associated labour and equipment costs. Vines that do not require sprays also have the potential for resulting wine to be marketed as produced from an environmental friendly vineyard.

The variation in yield and wine flavor suggests that it may be possible to identify high yielding mildew resistant selections that can produce diverse wine styles. High yields per hectare with the desired fruit composition will be of increasing importance in the future with a likely scenario of scarce resources (land and water) and climate change requiring growers to maximize returns per unit land and/or megalitre of water.

Selections that have high yields and reach maturity early would be of benefit to industry as they would require less resources (water, management) from budburst to harvest and would also be likely to avoid damaging weather events (heat stress, hail & rain damage) late in the season. For wineries or regions requiring greater control of vine vigour the regulation of water and/or the use of low vigour rootstocks could be used for high vigour, high yielding scion selections.

Adoption of new varieties has in the past been relatively slow in the wine industry compared to other crops and the dried and table grape industries. This is in part historical resulting in the global dominance of only a few “international varieties” and associated marketing. However the fickle nature of the wine market with varieties going in and out of fashion (e.g. Chardonnay, Sauvignon blanc) suggests that there is an opportunity to develop and market a new variety. Low alcohol, low carb wines are increasing in popularity in the UK market especially with the recent changes to tax on alcohol in wine. As demonstrated in this report it is possible to produce a light dry red wine with low alcohol using red flesh selections that have been harvested early. Another option for the adoption of these new varieties is use in blending or in wines that do not require a variety name on the label. There are advantages to not having a variety name on the label as the varieties and blend can be varied over time under a single label.

Adoption of new varieties is often helped by regional trials where growers can see the performance of new varieties in their own region. Development of partnerships to assist in the assessment of new varieties in different regions would be of value to both researchers and industry. These trials would be particularly useful in assessing the response to different disease pressures in the different regions and the associated yield and wine flavour.

Appendix 1 Communication

Fernandez L, Chaib J, Martinez-Zapater JM, Thomas MR, Torregrosa L (2013) Mis-expression of a PISTILLATA-like MADS box gene prevents fruit development in grapevine. Plant Journal 73: 918-928

Cabezas J, Ibanez J, Lijavetzky D, Velez D, Bravo G, Rodriguez V, Carreno I, Jermakow A, Carreno J, Ruiz-Garcia L, Thomas M, Martinez-Zapater J (2011) A 48 SNP set for grapevine cultivar identification. BMC Plant Biology 11: 12

Dry IB, Feechan A, Anderson C, Jermakow AM, Bouquet A, Adam-Blondon AF, Thomas MR (2010) Molecular strategies to enhance the genetic resistance of grapevines to powdery mildew. Australian Journal of Grape and Wine Research **16:** 94-105

The project was publicised in the GWRDC publication R&D@work in February 2011 "Breeding mildew resistance into vines".

Industry presentations
A presentation was given to Regional Program members, GWRDC's regional extension and adoption program on 2 May 2013.

Conference presentations
Thomas MR (2011) New grapevines from old - the power of molecular genetics. Plenary Session of the Crush 2011 meeting, 28-30 September 2011.

Crystal Sweetman, David Contreras-Pezoa, Yong Jia, Emily Higginson, Kathleen L Soole, Mark Thomas, and Christopher C.M.F Ford (2012) Organic acid metabolism of *Vitis vinifera* fruit. ComBio 2012, 23-27 September 2012, Adelaide, SA

Emily G. Higginson, Mark R. Thomas, Christopher M. Ford (2013) Discovering and characterising genes involved in tartaric and malic acid metabolism. The 15th Australian Wine Industry Technical Conference. Australian Wine Industry Technical Conference Inc., 13-18 July 2013, Sydney, NSW

Ian B. Dry, Angela Feechan, Mark. R. Thomas (2013) New vines for new times. The 15th Australian Wine Industry Technical Conference. Australian Wine Industry Technical Conference Inc., 13-18 July 2013, Sydney, NSW

Appendix 2 Intellectual Property

1. SNP markers used for marker assisted selection and genome wide analysis. SNP primer designs for SNP analysis used the Sequenom MALDI-TOF MS detection system. The primer designs and marker information are held by CSIRO and are not included in this report.

2. SNP DNA profiles of selected varieties. The DNA profiles are held by CSIRO and only a few included in this report.

3. New white and red selections resistant to downy and powdery mildew producing wines with diverse wine flavour profiles.

4. A mutagenized microvine population.

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Industry collaborators: Tony Hooper and Rebekah Richardson (Premium Wine Brands, Orlando Wines), Sam Wigan and Louisa Rose (Yalumba) and Corey Ryan (McWilliam's Wines).

Appendix 5 Other Material and Raw Data

Raw data is kept at CSIRO Plant Industry.